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PURIFICATION AND PROPERTIES OF *Pseudomonas aeruginosa* BACTERIOPHAGE SDI

by

PETER D. SHARGOOL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR

THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

APRIL, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "PURIFICATION AND PROPERTIES OF *Pseudomonas aeruginosa* BACTERIOPHAGE SDI", submitted by Peter D. Shargool in partial fulfilment of the requirements for the degree of Master of Science.

ACKNOWLEDGEMENTS

The author is indebted to Dr. E.E. Townsend who supervised the work contained in this thesis.

He would also like to thank the following people for their help and encouragement:

Dr. B.G. Lane, for a number of suggestions, including the enzymic method of base analysis, and for help in carrying this out.

Dr. T. Yamamoto, for supplying many electron microscope and other pictures and for many interesting conversations.

Dr. J.N. Campbell, who supplied the strains of _Pseudomonas aeruginosa_ used in this work, and encouragement in its early stages.

Mrs. Ursula Von Meydall, for assistance with the statistical analysis.

Mr. B.D. McLennan, who donated quantities of snake venom phosphodiesterase.

Mrs. A. Bloom, for the typing of the final copies of this thesis.

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ABBREVIATIONS

A	=	adenine
C	=	cytosine
c.f.u.	=	colony forming unit
CM-	=	carboxymethyl-
DEAE-	=	diethyl amino ethyl
DNA	=	deoxyribonucleic acid
DNAse	=	deoxyribonuclease (pancreatic)
5'-dAMP	=	deoxyadenosine-5'-phosphate
5'-dCMP	=	deoxycytidine-5'-phosphate
5'-dGMP	=	deoxyguanosine-5'-phosphate
5'-dTMP	=	deoxythymidine-5'-phosphate
EDTA	=	ethylenediamine tetraacetate
G	=	guanine
N	=	nitrogen
P	=	phosphorus
p.f.u.	=	plaque forming unit
phage	=	bacteriophage
RNA	=	ribonucleic acid
RNAse	=	ribonuclease (pancreatic)
T	=	thymine
UV	=	ultraviolet

Chapter 1.

Introduction.

One of the first studies of a *Pseudomonas* bacteriophage was made by Hadley in 1924, only a few years after the discovery of the existence of phages by Twort and d'Herelle.

Hadley reported finding a lytic principle associated with one of two strains of *Pseudomonas aeruginosa* isolated from a hospital patient. The lytic principle was present in filtrates obtained by passing a broth culture of the strain with which the phage was associated through a Berkfeld candle. The filtrate caused lysis to occur in certain strains of *P. aeruginosa* and *P. fluorescens*, but not in other bacterial species. Hadley also showed that the concentration of the lytic principle increased at the expense of bacteria of a sensitive strain.

The pathenogenicity of *P. aeruginosa* has motivated a number of studies into the use of phages as agents for typing strains of this organism isolated from hospital patients. A recurring theme in much of this work is the prevalence of the lysogenic condition in many of the strains isolated from natural sources. Warner, in 1950, isolated at least 25 phages from 68 strains of *P. aeruginosa*. Feary and Fisher (1963a) found that 95 strains of *P. aeruginosa* isolated from hospitals in New Orleans were all lysogenic for one or more phages. Holloway (1960) found that 45% of the 200 strains he isolated from hospitals in Melbourne were lysogenic.

In a later study Holloway et al (1960) divided phages isolated from amongst 81 strains of P. aeruginosa into 6 groups according to their serological cross reactions. The members of each group were found to correspond in properties other than their antigenic type. Phages of one group were readily produced after induction by U.V. light of the bacterial strains lysogenic for them. Three other groups were characterized by the transducing properties of the phages. Another group contained phages of a virulent type which were able to lyse broth cultures of sensitive bacterial strains completely, no lysogenic bacteria being produced.

Electron microscope studies by Bradley (1963) and Feary and Fisher (1964) have revealed that P. aeruginosa phages are characterized by a wide variety of morphological forms and sizes. Many of these resemble the characteristic forms of different E. coli phages.

Only recently have reports of biochemical studies on P. aeruginosa phages appeared. A minute phage, 7S, having a diameter of 25 mμ was found associated with a strain of P. aeruginosa by Feary and Fisher (1963b, 1964). The nucleic acid component contains adenine, guanine, cytosine, and uracil in approximately equal quantities and ribose. A brief description of a DNA-containing phage was reported by Grogan and Johnson in 1964. The phage particle contains approximately 1.2×10^{-16} gm of DNA which appears to have a highly ordered structure: guanine and cytosine comprise 55% of the DNA bases. The phosphorus content was 8.7% of the DNA.

More extensive biochemical studies should permit a comparison to be drawn between phages and phage-host relation-

ships of Pseudomonas species and those of other bacterial species. This thesis describes the purification and some morphological and chemical properties of a bacteriophage virulent for a strain of P. aeruginosa. These properties are compared as far as possible with those of other phages possessing similar morphological properties, in Chapter 4.

Chapter 2.

Materials and Methods.

Chapter 2.

A. Biological Materials and Methods.

1. Host bacterium - P. aeruginosa B71.

The organism used in these studies was *Pseudomonas aeruginosa* strain B71, obtained from the Microbiology Department, where it was isolated from raw milk in a classroom experiment.

2. Growth media.

a. Defined medium.

Preliminary studies of growth conditions for the bacterium indicated that it would grow well in the salts glucose medium described by Jacob (1952). The composition of this medium is as follows:-

KH_2PO_4	13.6 gm
$(NH_4)_2SO_4$	2.0 gm
$MgSO_4.7H_2O$	0.2 gm
$Ca(NO_3)_2$	0.1 gm
$FeSO_4.7H_2O$	0.5 mg
water to 1000 ml	

The pH was adjusted to 7.0 with KOH. Sterile 30% glucose solution was added to the autoclaved salts solution, to give a final concentration of 3 gm per liter. In practice the above medium was modified slightly by using $CaCl_2$ instead of $Ca(NO_3)_2$ and by using the following stock solutions:-

$(NH_4)_2SO_4$	1.5 M	10.0 ml/liter
$FeSO_4$	0.0028 M	1.0 ml/liter
$CaCl_2$	0.01 M	1.0 ml/liter
$MgSO_4$	0.1 M	10.0 ml/liter

The KH_2PO_4 was still added as described above.

b. Nutrient broth.

Nutrient broth was routinely used as a growth medium for seeding cultures, the formula being as follows:-

Nutrient broth (Difco) 10 gm per liter.

This was dissolved in water, and the solution buffered with sodium phosphate to give a final pH of 7.0 and a phosphate concentration of 0.01 M. Sterile 30% glucose solution was added to give a final concentration of 0.3%.

c. Solid agar.

Nutrient agar for plates and slants was prepared as follows:

Bacto agar (Difco)	15 gm
Nutrient broth (Difco)	10 gm
NaCl	5 gm
Glucose	1 gm
water to 1000 ml	

d. Semi-solid agar.

Nutrient agar for use in phage titrations was prepared in the same manner as the solid agar except that 6.5 gm of agar per liter were added.

3. Standard diluent.

Physiological saline containing 0.01 M sodium phosphate pH 7.0 was used for the dilution of bacterial cultures and phage preparations prior to assay.

4. Propagation of *P. aeruginosa* B71.

In order to ensure that the host strain was pure, strain B71 was grown in nutrient broth, and then spread on a nutrient agar plate to give single colonies. A single colony was picked and grown in nutrient broth, then spread on a nutrient agar plate; the process of picking single colonies and producing cultures from these was repeated a number of times. To provide stock cultures of *P. aeruginosa* B71 and to ensure the viability of these cultures, the bacteria were maintained by spreading a loopful of an early log phase nutrient broth culture on a nutrient agar plate. A single colony was picked to inoculate nutrient broth which when cultured to a suitable stage was used as seed for phage titrations or to inoculate liquid cultures.

5. Growth characteristics of *P. aeruginosa* B71.

Growth rates were measured in both nutrient broth and in defined medium, using a growth vessel constructed from a 250 or 500 ml conical flask. A 25 mm tube was sealed to the mouth of the flask and a side arm sealed to the flask so that samples could be withdrawn. Turbidity was measured by inverting the flask so that the medium inside was contained in the 25 mm tube, which served as a cuvette for use in the Coleman Junior spectrophotometer. The turbidity was determined at 650 mμ for nutrient broth cultures and 440 mμ for cultures grown in the defined medium. Appropriate blanks were used in each determination.

FIG 4.

The increase in viable count (c.f.u./ml.) with time (hours) of P. aeruginosa B 7I growing in defined medium at 37°C.

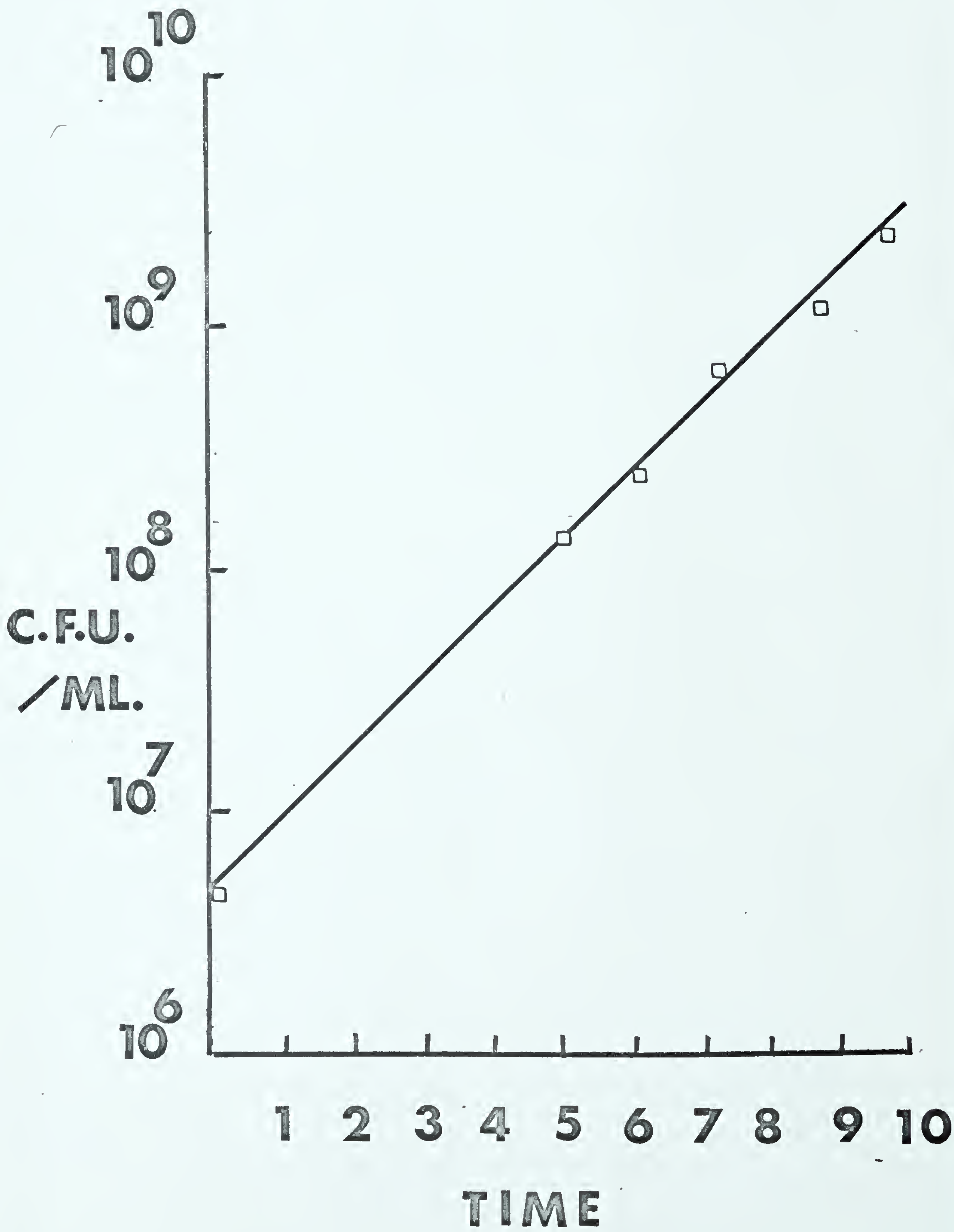


FIG. 3.

Relationship of viable count (c.f.u./ml.) to turbidity measured at 440 mu of P. aeruginosa B7I growing in defined medium at 37°C.

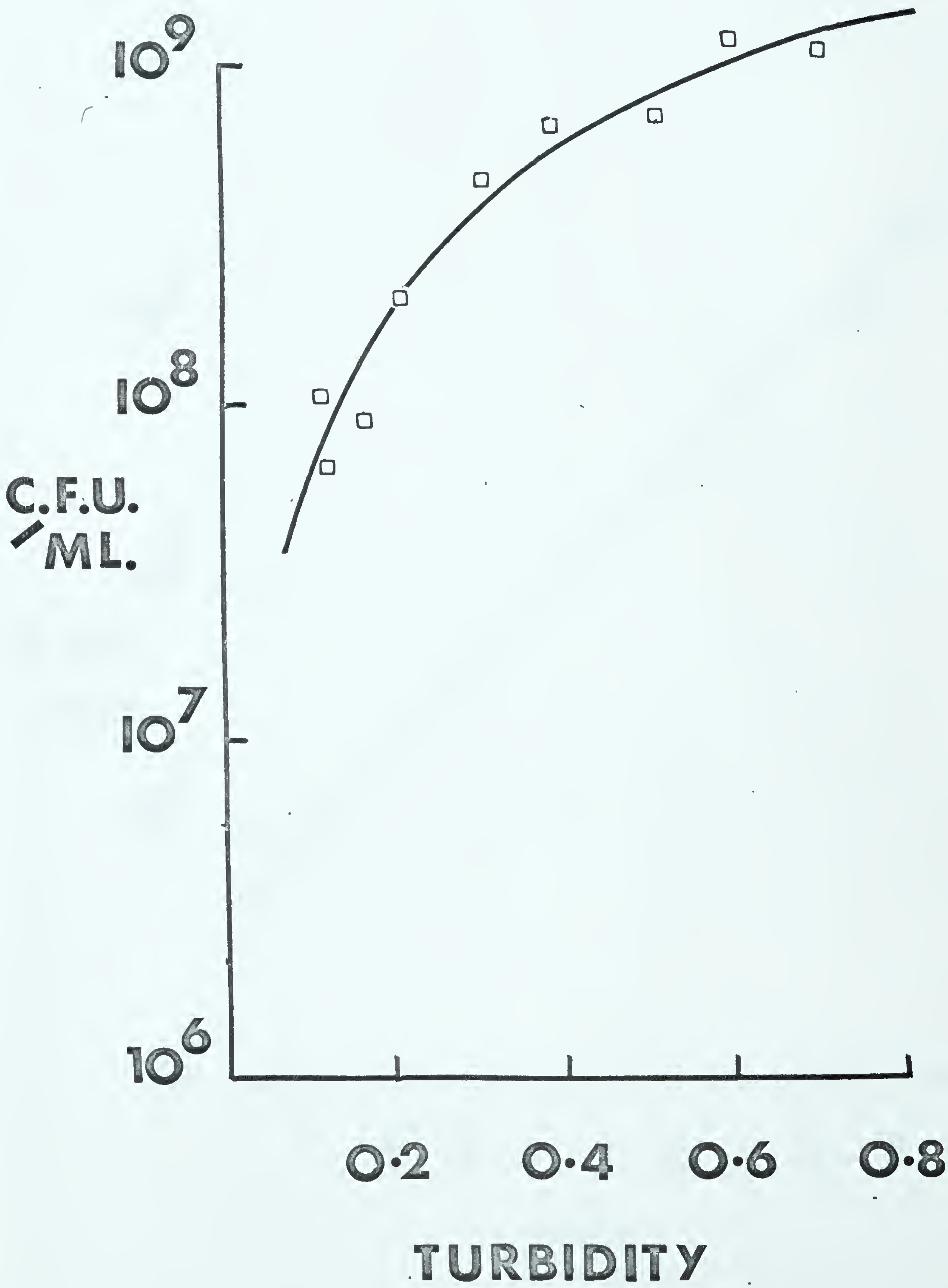


FIG 2.

The increase in viable count (c.f.u./ml.) with time (hours) of P. aeruginosa B7I growing in nutrient broth at 37°C.

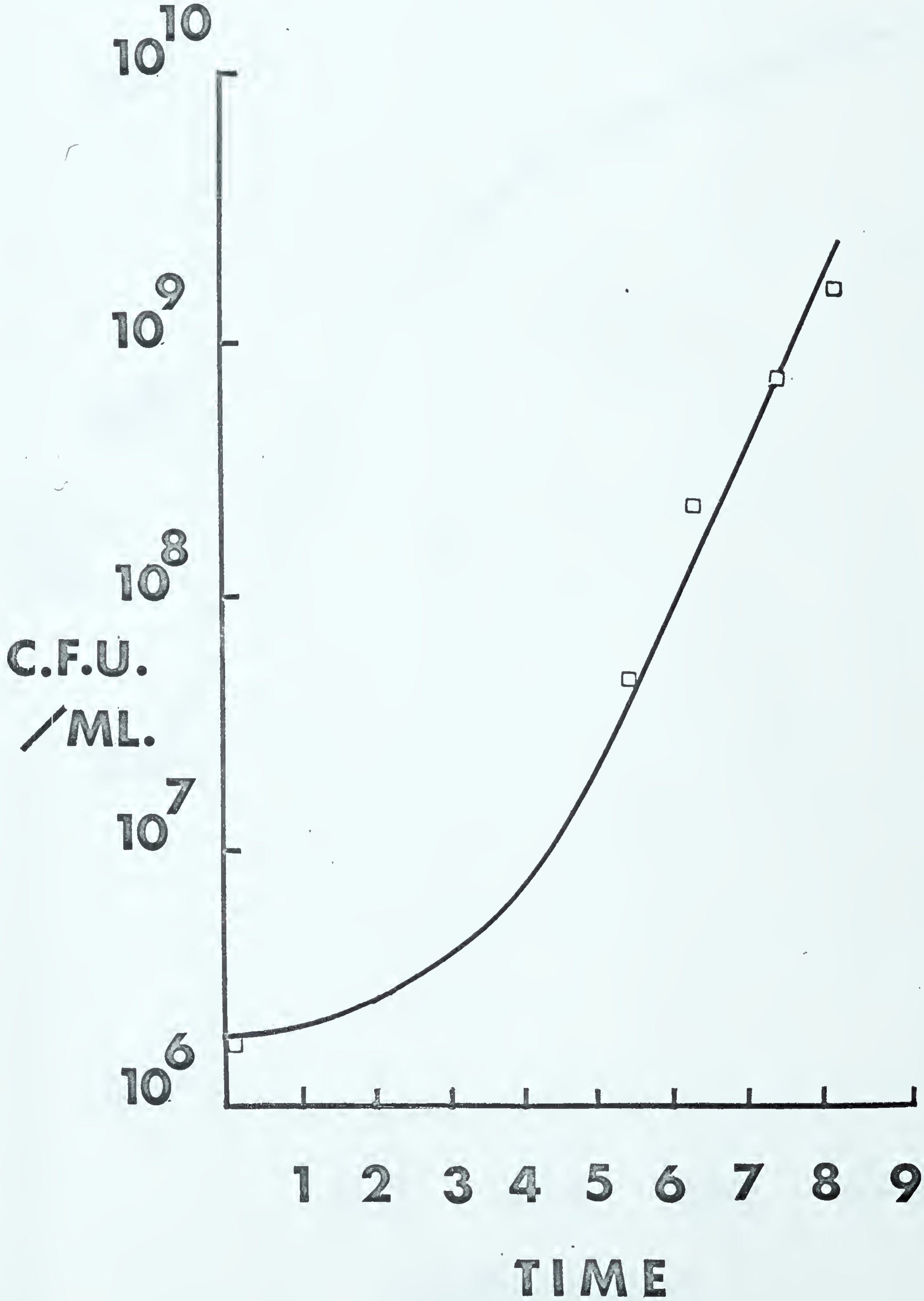
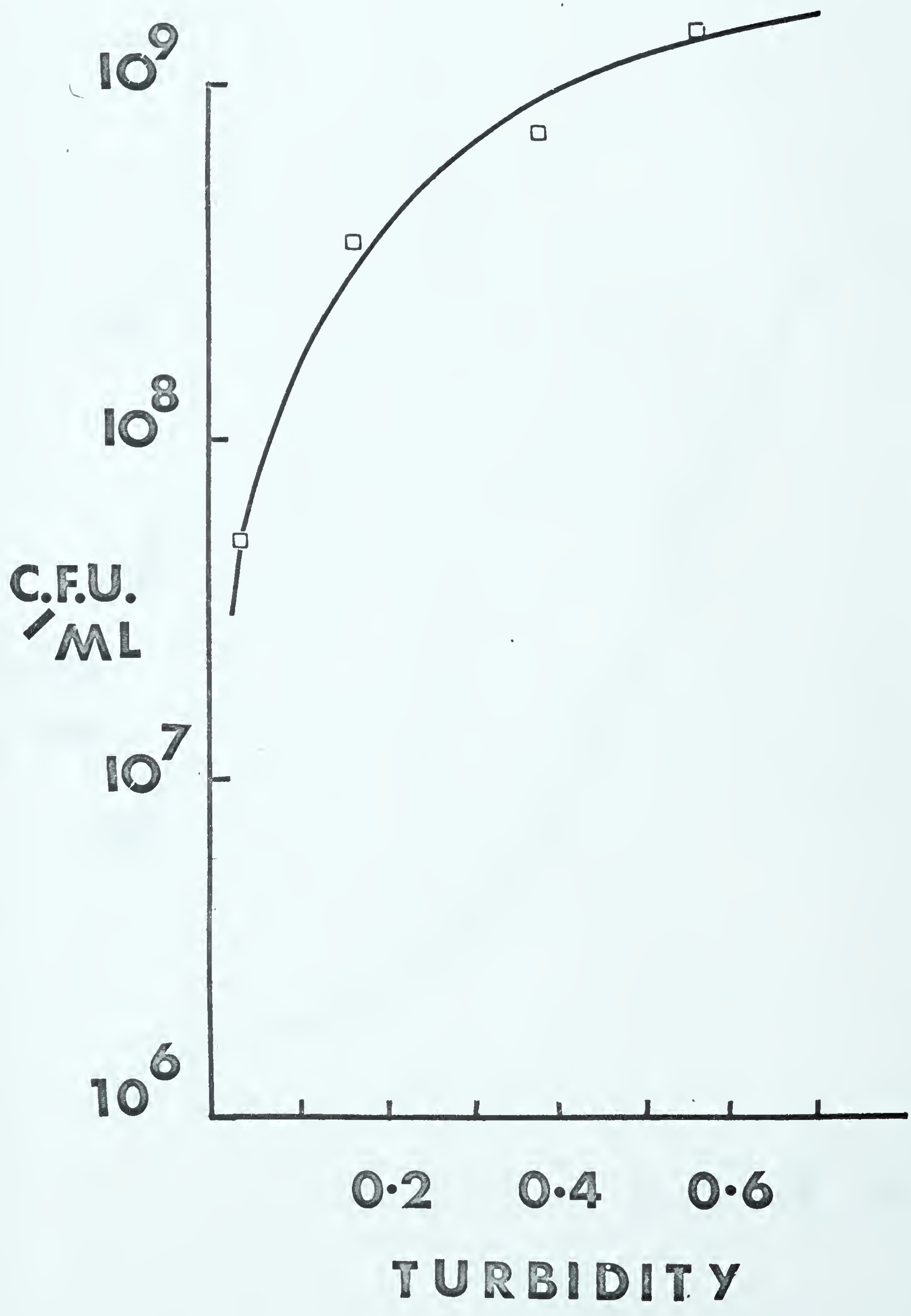


FIG I

Relationship of viable count (c.f.u./ml.) to turbidity measured at 650 mu of P. aeruginosa B71 growing in nutrient broth at 37°C.



a. Growth in nutrient broth.

A 30 ml aliquot of nutrient broth was inoculated with an aliquot of an overnight nutrient broth culture and incubated at 37°C in a shaking water bath. The turbidity of the culture was measured at hourly intervals and aliquots were withdrawn, diluted, and plated for estimation of the viable cell count. The correlation between turbidity and viable count, and between viable count and time, are depicted in Figs. 1 and 2. The doubling time can be estimated from Fig. 2 to be about 30 mins.

b. Growth in defined medium.

A similar procedure was followed for the correlation of turbidity with viable cell count and viable cell count with time in the defined medium. These are depicted in Figs. 3 and 4. The doubling time can be estimated from Fig. 4 to be about 70 mins.

6. Agar layer method of phage titration.

The method used to assay phage was based on that of Adams (1959). Semi-solid agar was melted, cooled to 45°C, and 3 ml aliquots dispensed into sterile test tubes kept in a water bath at 45°C. Aliquots of 0.2 ml of a nutrient broth culture of *P. aeruginosa* B71, the turbidity of which was 0.05 to 0.25 Coleman units, were added to the tubes of agar. This turbidity range was found to give fairly consistent titration results in experiments performed on a standard phage suspension. The phage suspension was serially diluted

in saline phosphate buffer and a 0.1 or 0.2 ml aliquot of the appropriate dilution was added to a tube of soft agar seeded with bacteria. The contents of the tube were mixed and quickly spread over the surface of an agar plate. The plates were incubated at 37°C overnight, and the plaques that developed were counted and corrected for dilution.

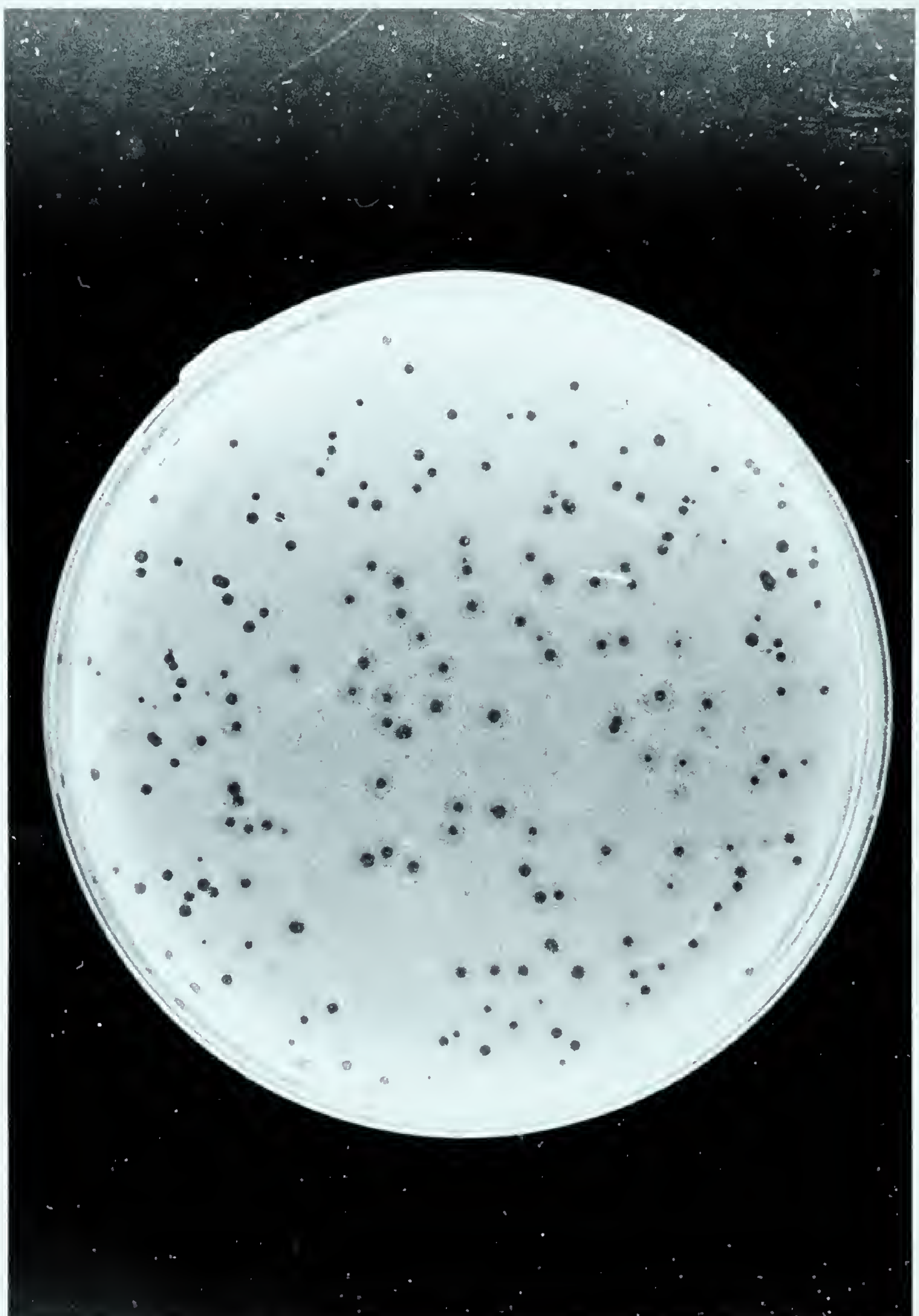
7. Isolation of bacteriophage SDI.

Four strains of *P. aeruginosa* - B71, PAE-2, 24', and 20' - were obtained from Dr. Campbell of the Microbiology Department. With the exception of strain 20' they were found to grow satisfactorily in the defined medium of Jacob (1952) which was chosen arbitrarily for this purpose. Samples of raw and effluent sewage were obtained from the Edmonton city sewage plant, and centrifuged at 4,500 r.p.m. for 5 mins in the International centrifuge. Aliquots of the supernatant of the raw sewage sample were added to the log phase cultures of strains B71, PAE-2, and 24'. The cultures were incubated at 37°C with shaking overnight. The following day the cultures were lysed with chloroform, centrifuged, and the supernatants titrated against nutrient broth cultures of the bacterial strains originally used for the production of each particular lysate. Plaques were produced with strain B71 only. These were of three kinds: small without a halo, medium with a halo, and large with a halo.

Since all four strains grew well in nutrient broth, the experiment was repeated using effluent sewage. Again B71 was the only strain yielding plaques.

Figure 5

Plaque morphology of phage SDI



Two additional strains, MAC 264 and 489, also strains B71, 24', and PAE-2, were grown to log phase in the defined medium, and incubated with aliquots of a freshly obtained sample of effluent sewage which had been passed through a Millipore filter to remove sewage bacteria. After incubation the cultures were shaken with chloroform, centrifuged, and the supernatants titrated against the corresponding bacterial strains as before. Plaques of the three types already described were produced on both MAC 264 and B71 strains. When each of the plaque types produced from either of the two sewage samples was stabbed and replated, each gave rise to a mixture of plaques, small, medium, and large. Thus it was assumed that only one plaque type had been isolated.

In experiments to determine whether MAC 264 or B71 would be the most satisfactory host strain, it was found that the yield of phage from MAC 264 seemed to be less than from B71. The reason for this was not determined however, and strain B71 was chosen as the host. The morphology of the phage was revealed at this stage in negatively stained preparations from a plaque examined in the electron microscope by Dr. T. Yamamoto of the Microbiology Department.

Chapter 2.

B. Chemical and Physical Methods.

1. Electron microscopy.

Electron microscope studies were performed at times when it was desired to check the purity of phage preparations. A few ml of preparations titering at least 1×10^{12} p.f.u. per ml in 1% ammonium acetate were given to Dr. T. Yamamoto who performed negative staining in phosphotungstic acid and subsequent electron microscopy on these preparations. Measurements of the dimensions of the phage were made from electron microscope pictures.

2. Nucleic acid extraction.

The method of Mandell and Hershey (1960) was used to extract DNA from purified phage preparations. Aliquots, 10 ml of phage preparations titering between 8×10^{11} and 2×10^{12} p.f.u. per ml were extracted three times with 10 ml of water saturated distilled phenol: the phenol layers were washed successively with 10 ml of 0.1 M NaCl-phosphate buffer. The combined aqueous layers were extracted once with cold ether, the DNA was precipitated with 2 volumes of cold isopropanol and the precipitate washed twice with cold 95% ethanol. The DNA precipitate was partially air dried, and then dissolved in distilled water or buffer, as required.

3. DNA estimation.

DNA in whole phage preparations titering no less than

10^{11} p.f.u. per ml and in stock solutions of DNA was estimated by the diphenylamine method of Burton (1956). This method involved hydrolysing aliquots of from 0.5 to 2.0 ml of the DNA-containing samples for 15 mins at 70°C in 0.5 N perchloric acid, and estimating the purine bound deoxyribose liberated by treatment with diphenylamine reagent. A standard deoxyguanosine solution (prepared from Calbiochem grade A deoxyguanosine), was used to prepare a standard curve each time the assay was performed. Using the conditions described by Burton, 1 to 2 μg of DNA can be measured. The standard curve was linear for samples containing less than 15 μg of deoxyguanosine per ml.

4. Phosphorus estimation.

Phosphorus estimations were performed using the micromethod of Ames and Dubin (1960). A 5 ml sample of purified phage in 1% ammonium acetate, titering approximately 1 x 10^{12} p.f.u. per ml, was sedimented at 30,000 r.p.m. in the No 40 rotor of the Model L Spinco ultracentrifuge. The pellet was resuspended in 0.5 ml of 1% ammonium acetate. Determinations of the phosphorus content of whole phage were carried out on this suspension. Total phosphorus was determined on the supernatant left after precipitating the phage with an equal volume of cold 10% trichloroacetic acid and sedimenting the precipitate in a clinical centrifuge. Aliquots of 5 to 40 μl of the whole phage preparation and 50 to 100 μl of the suspending medium after removal of the

phage were ashed with $Mg(NO_3)_2$ and the resulting residue was dissolved in 0.5 N HCl. A phospho-molybdate complex was formed and the amount present estimated at 820 mμ using the Beckman DU spectrophotometer. Aliquots of a standard phosphorus solution prepared from potassium dihydrogen phosphate ("Baker analysed" reagent grade) were used to prepare a standard curve each time the assay was performed. The standard curve was linear over the range of concentrations used which was from 0.06 to 2.4 μg of phosphorus. Under the conditions described above, 1% of non-phage phosphorus could be determined in the suspending medium.

5. Nitrogen estimation.

The nitrogen content of whole phage, and of the protein and nucleic acid fractions of the phage, was determined by the method of Johnson (1941). Aliquots of 0.25 to 1.0 ml of a phage preparation titering 1.5×10^{12} p.f.u./ml were taken for estimation of the total nitrogen content of phage SDI. In order to determine the nitrogen content of the protein and nucleic acid components, 1.0 ml aliquots of the phage preparation were precipitated with 4 vols of cold 10% trichloroacetic acid. The nucleic acid fraction was isolated by extracting the precipitate two times with hot 5% trichloroacetic acid (Schneider, 1945). The precipitated protein was dissolved in 0.1 N NaOH and nitrogen determinations were performed on this fraction and on the hot trichloro-

acetic acid fraction. Digestion of the samples was carried out with H_2SO_4 and $CuSeO_3$ at 120°C for 16 hours, the digestion being complete in this period of time. A mercuri-ammonium complex was formed and stabilized with gum ghatti: the amount formed was determined at 490 mμ in the Beckman DU spectrophotometer. Aliquots of a standard ammonium chloride solution ("Baker analysed" reagent grade) were used to prepare a standard curve each time an estimation was carried out. The standard curve was linear between 2.0 and 45 μg of nitrogen.

6. DNA base analysis.

a. Perchloric acid hydrolysis.

Purified phage preparations were hydrolysed with 72% perchloric acid, according to the method of Marshak and Vogel (1951). A 10 ml aliquot containing 1×10^{12} p.f.u. per ml was sedimented in the Model L Spinco ultracentrifuge, and taken to dryness *in vacuo*. 40 μl of 72% perchloric acid were added, an atmosphere of nitrogen introduced and the stopper of the hydrolysis tube sealed in place with tape. After hydrolysis at 100°C for 40 mins , 40 μl of distilled water were added, and a 15 μl aliquot of the resulting suspension was applied to a sheet of Whatman No. 1 chromatography paper. This was subjected to descending chromatography using aqueous isopropanol (65% isopropanol) made 2 N with HCl (Wyatt, 1951). The bases were located with U V light and eluted in 0.1 N HCl. Corresponding blank areas of chromatograms were eluted in 0.1 N HCl for use as blanks

in the spectral analysis. The bases were identified by comparison with known standards, by their migration in the chromatographic system, and by their U V spectral properties.

b. Enzymic hydrolysis.

A DNA aliquot equivalent to 200 μg of DNA was precipitated with 2 volumes of cold isopropanol and washed with cold 95% ethyl alcohol. After dissolving the precipitate in 200 μl of distilled water, 2 μl of 0.1 M $MgSO_4$ and 100 μl of a solution containing 0.2 mg per ml of DNAse (Hurst, Marko and Butler, 1953) were added. This solution was incubated at 37°C for 60 mins , then 60 μl of ammonium formate buffer pH 9.0 and 200 to 400 μl of snake venom phosphodiesterase (free of phosphomonoesterase activity) were added. The exact amount of enzyme added was determined by its activity. The mixture was incubated at 37°C for 20 hours, and then subjected to 2-dimensional paper chromatography according to Singh and Lane (1964). The solvent used for the first dimension was composed of 80 volumes of 95% ethanol and 20 volumes of water, and for the second dimension, of 80 volumes of saturated ammonium sulfate and 2 volumes of isopropanol. The separated nucleotides were located with U V light, and eluted with 0.1 N HCl, as were corresponding blank areas of the chromatograms. The spectra of the pyrimidine nucleotides and the purine bases (produced from the corresponding nucleotides by hydrolysis in 0.1 N HCl) were obtained using the Bausch

and Lomb recording spectrophotometer. They were identified by comparison with known compounds.

c. Trace base analysis.

Aliquots containing 0.4 mg of phage DNA were hydrolysed with 0.3 ml of 90% formic acid for 60 mins at 175°C and the hydrolysates evaporated to dryness in vacuo (Wyatt and Cohen, 1953). The residues were dissolved in 30 μl of 0.1 N HCl and applied to Whatman No. 1 chromatography paper. Chromatography was carried out in a descending fashion in each of two solvent systems: 1) aqueous isopropanol (65% isopropanol) made 2 N with respect to HCl (Wyatt, 1951); 2) aqueous isopropanol (65% isopropanol) with the addition of a few mls of ammonia to the solvent in the bottom of the tank (Hershey et al, 1953). These solvent systems resolve 5-methylcytosine and 5-hydroxymethylcytosine respectively from adenine, guanine, cytosine and thymine. An amount of 5-methylcytosine equal to 2.0 μg in a 1.5 cm spot on a chromatogram would be detectable.

d. Glucose determination.

The method used to detect the presence of glucose in phage DNA was that of Takahashi and Marmur (1963). Aliquots containing 0.48 mg of phage DNA were hydrolysed for 1 hour in 1 N HCl, and then chromatographed on Whatman No. 1 chromatography paper. The solvent used for the separation was composed of 80 parts isopropanol and 20 parts of water: it was used in a descending fashion. The chromatogram was run for 16 hours, dried and sprayed with diphenylamine-aniline

reagent and developed in a hot oven. The method was found to be capable of detecting 10 μg of glucose in a 1.5 cm spot on a chromatogram.

e. Determination of DNA melting temperatures.

The method used to determine the melting temperature of the phage DNA was that described by Marmur and Doty (1962). A solution of 25 μg per ml of DNA in 0.15 *M* sodium chloride - 0.015 *M* sodium citrate, pH 7, was heated and the absorbancy at 260 mμ determined over the temperature range 25° - 100°C. The absorbancy determinations were performed in the Beckman DK spectrophotometer. The cuvette compartment was heated by circulating a water-ethylene glycol solution solution from a Haake thermostated water bath. Plastic covers were fitted on the quartz cuvettes used. The temperature of the DNA solution was read by means of a thermometer fitted through a hole in the plastic cover of the cuvette. The temperature corresponding to half the increase in absorbancy was designated the melting temperature or Tm. The guanosine and cytosine content of the DNA was then determined from the relationship derived by Marmur and Doty; $Tm = 69.3 + 0.41 (G + C)$ where (G + C) refers to the mole percentage of guanine and cytosine.

7. Buoyant density determination.

The buoyant density of the phage was determined on samples which were purified in a cesium chloride gradient. The preparation of this gradient is described in the section

dealing with the purification of the phage. One tube was selected from a purification experiment, a hole punctured in the bottom of the tube and drop fractions collected. Aliquots of 20 μl of each fraction were weighed and the specific gravity determined by comparison with the weight of an equal volume of water. A graph of specific gravity and of phage titer versus fraction number was constructed. The specific gravity of the phage peak was taken from this plot. Since centrifugation was carried out at approximately 5^{o}C and the specific gravity of the fractions was determined at room temperature, approximately 25^{o}C, corrections were made to obtain a value of the buoyant density at 5^{o}C. The density at 25^{o} was obtained by multiplying the specific gravity figure by 0.997, the density of water. The density at 5^{o}, equivalent to the buoyant density of the phage, was obtained by multiplying the density at 25^{o} by 1.007. This figure was calculated from data on the density of cesium chloride solutions at different temperatures in International Critical Tables.

Chapter 3.

Results.

FIG 10.

Experiment 5.

The increase in phage titer with time (hours) in defined medium. The initial concentrations of p.f.u. and c.f.u. were 5 x 10^6 and 6 x 10^8 per ml respectively, representing a multiplicity of 10^{-2}.

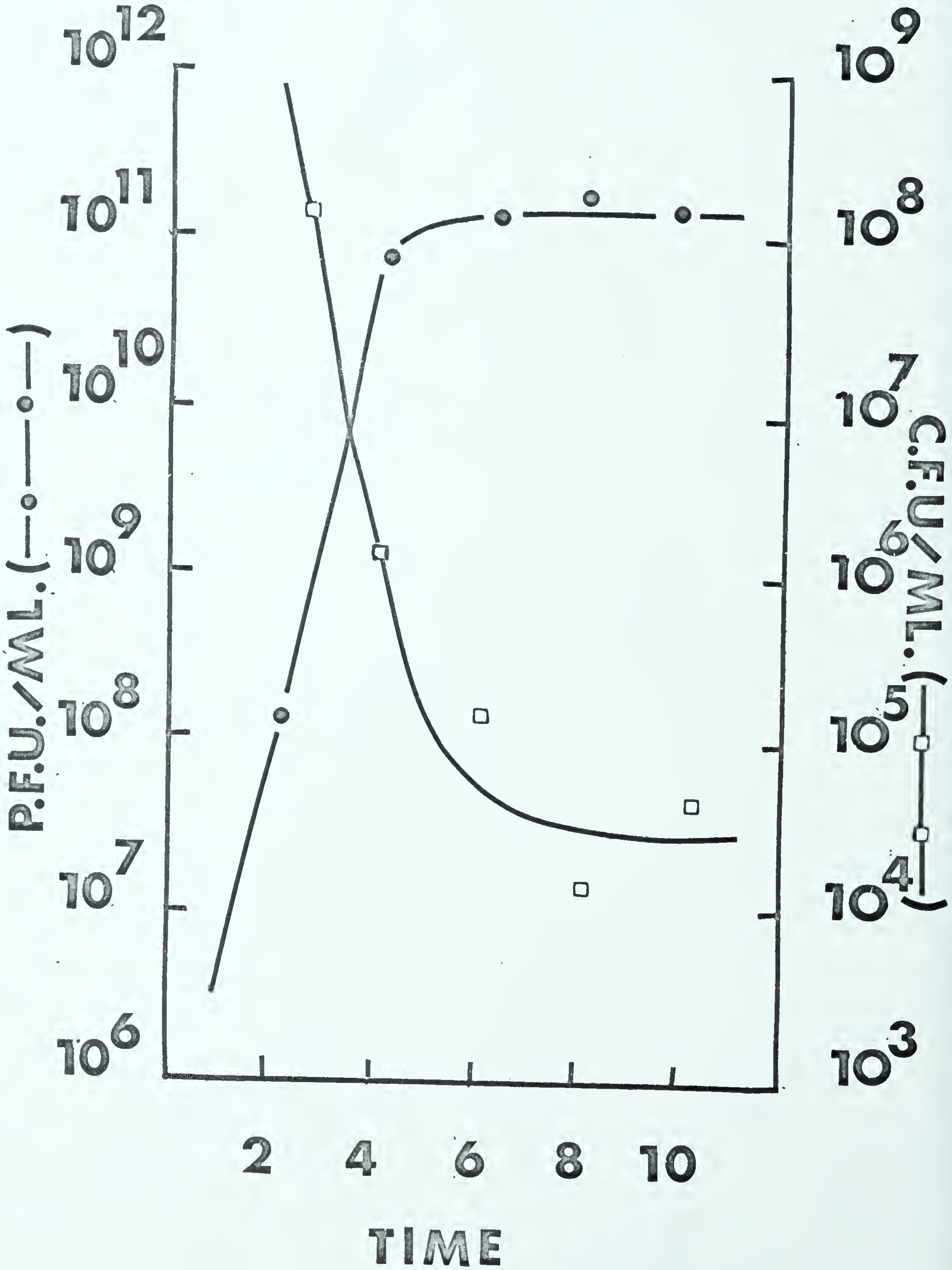


FIG 9.

Experiment 4.

The increase in phage titer with time (hours) in defined medium. The initial concentrations of p.f.u. and c.f.u. were 5 x 10^5 and 6 x 10^8 per ml respectively, representing a multiplicity of 10^{-3}.

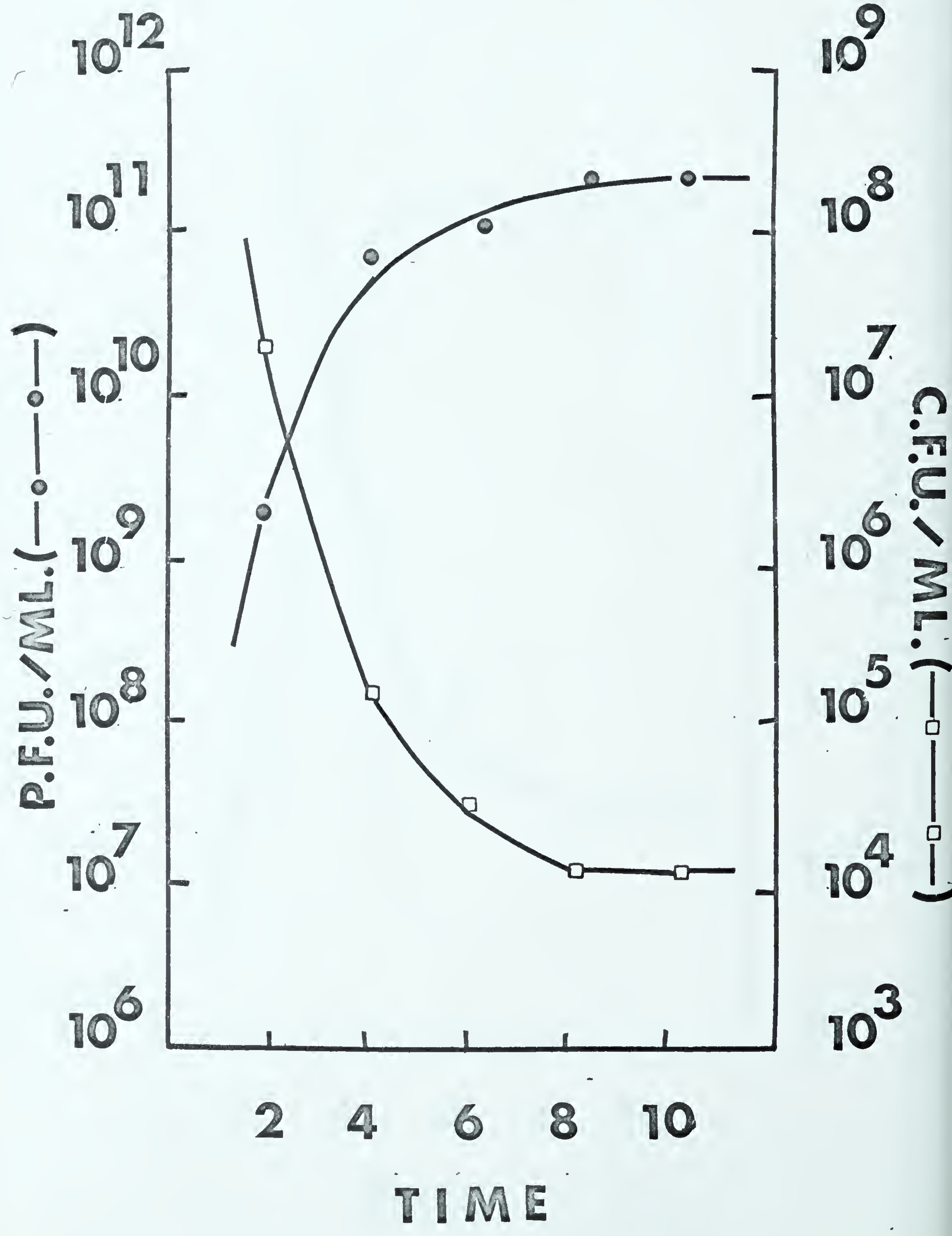


FIG 8.

Experiment 3.
The increase in phage titer with time (hours) in defined medium. The initial concentrations of p.f.u. and c.f.u. were 5 x 10^6 and 6 x 10^7 per ml respectively, representing a multiplicity of 10^{-1}

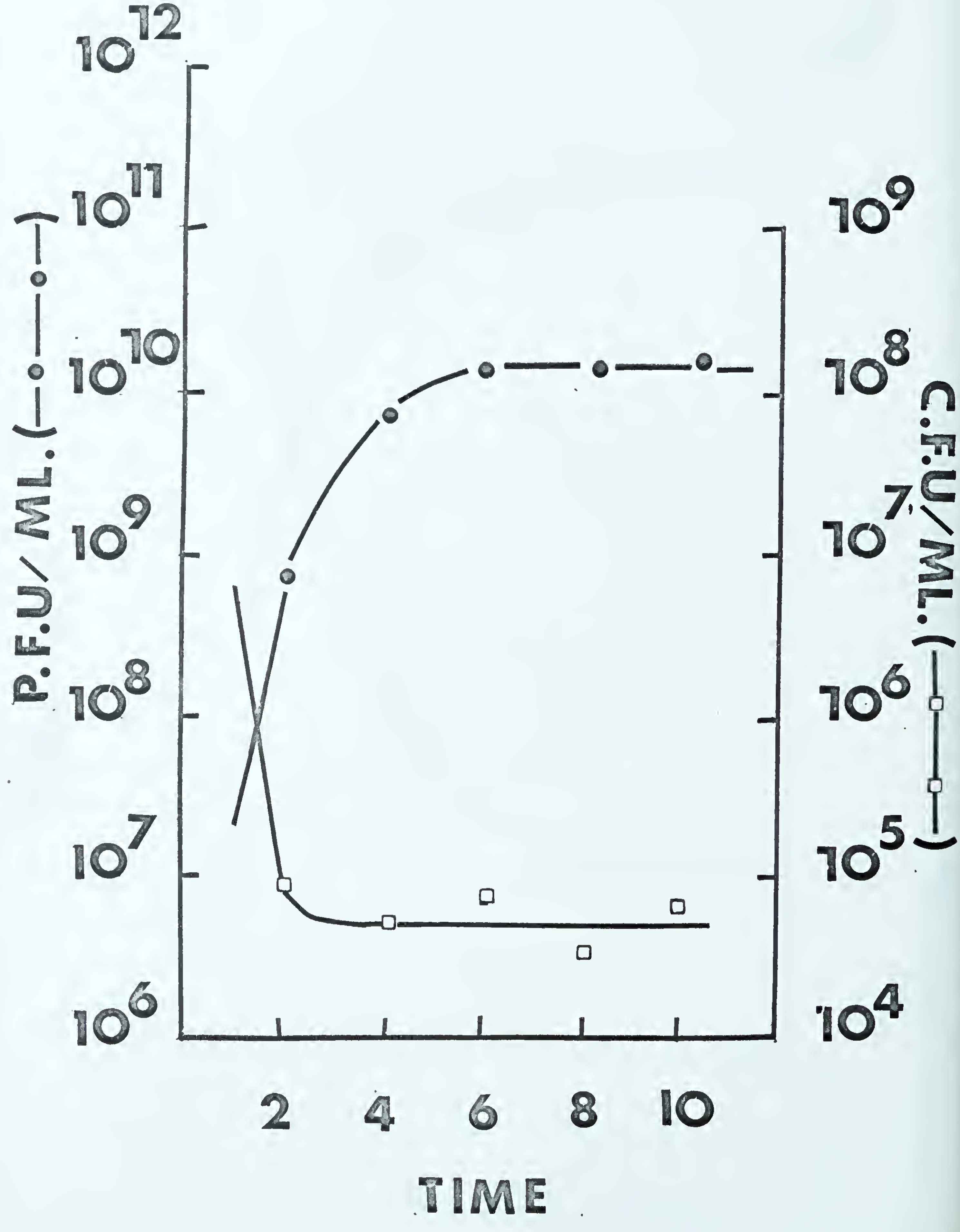


FIG 7.

Experiment 2.

The increase in phage titer with time (hours) in defined medium. The initial concentrations of p.f.u. and c.f.u. were 5 x 10^5 and 6 x 10^7 per ml respectively, representing a multiplicity of 10^{-2}

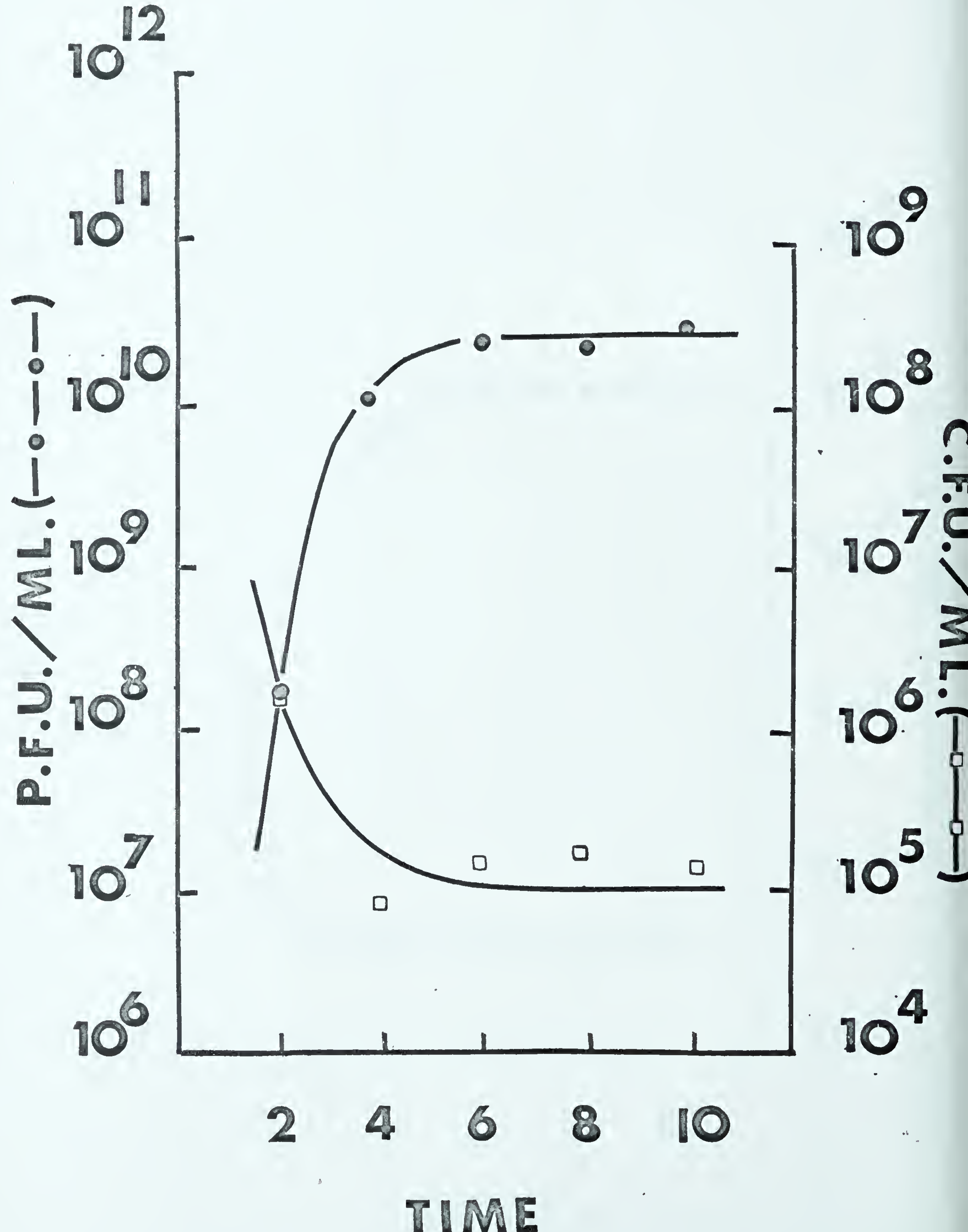
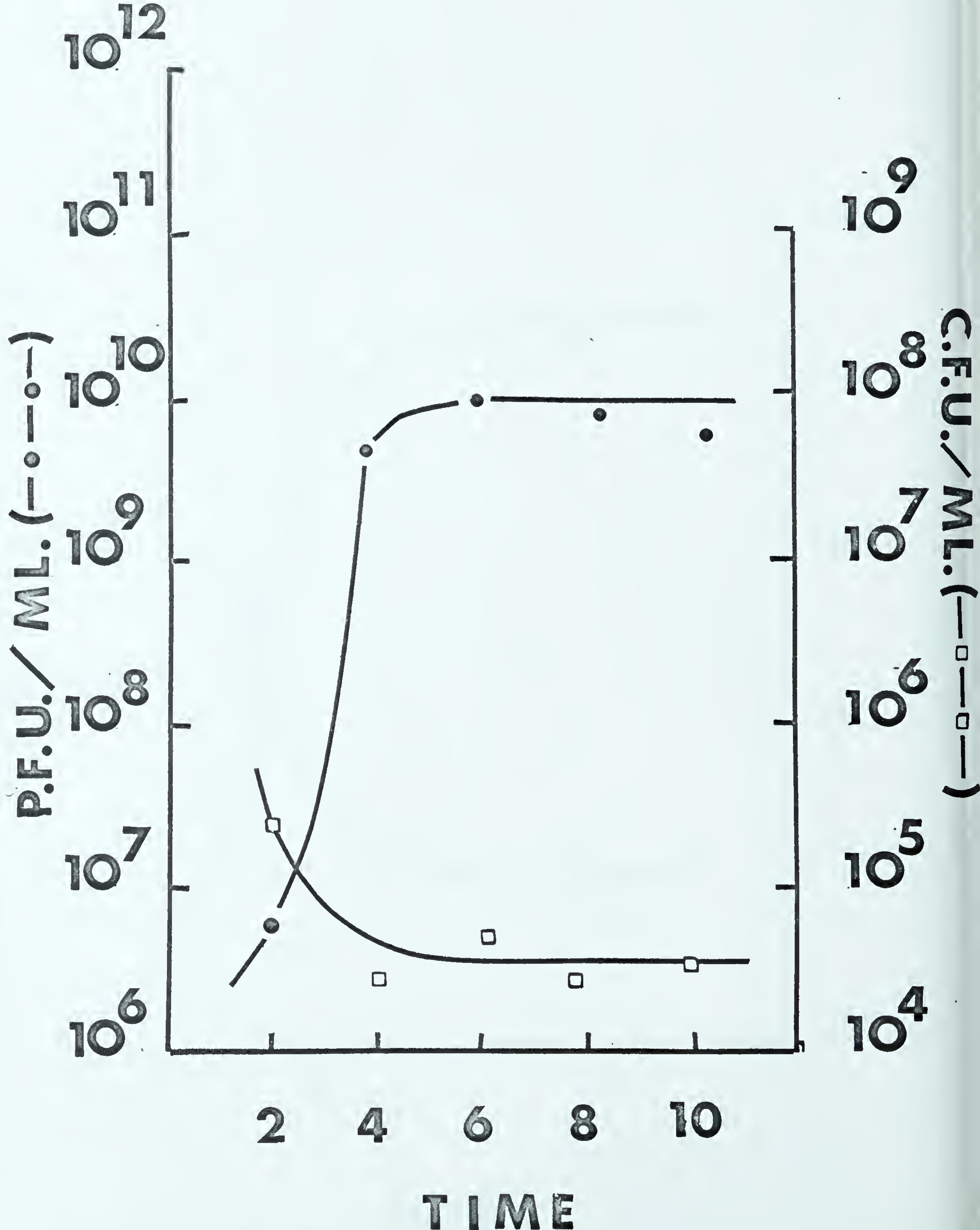


FIG 6.

Experiment I.

The increase in phage titer with time (hours) in defined medium. The initial concentrations of p.f.u. and c.f.u. were 5×10^5 and 6×10^6 per ml respectively, representing a multiplicity of 10^{-1}



Chapter 3.

A. Production and Purification of Lysates.

1. Production of lysates.

Analytical studies are made possible only if large amounts of phage are available. It is therefore desirable to obtain the phage in as large a yield as possible since losses may occur during purification. The conditions whereby optimal yields of phage are produced from liquid cultures were investigated. The multiplicity of infection (that is the ratio of p.f.u. to c.f.u.) and the initial concentration of bacteria were varied in 5 experiments. These experiments were carried out in the following way.

An overnight nutrient broth culture of *P. aeruginosa* B71 was suitably diluted into 500 ml of defined medium to give the required c.f.u. Phage was diluted in saline-phosphate buffer and added to the bacterial culture to give the appropriate p.f.u. concentration. Samples were withdrawn at 2 hour intervals, diluted, plated for surviving bacteria and titered for phage. The results are shown in Figs. 6 to 10, which depict the rise in phage concentration and the fall in bacterial concentration over the time period of the experiment.

The highest yield of phage was found in experiment 4 (Fig. 9), where the final phage titer obtained was 2×10^{11} p.f.u. per ml. The multiplicity of plaque forming to colony forming units employed in this experiment was 5×10^{5} p.f.u.

per 6 x 10^8 c.f.u., a multiplicity of 10^{-3}. This multiplicity was employed in the production of subsequent lysates in the following manner. An aliquot of an overnight nutrient broth culture of bacteria was added to defined medium and equilibrated at 37°C to give a concentration of approximately 10^7 c.f.u. per ml. This culture was then incubated until the turbidity measured at 440 mμ indicated the presence of 1 x 10^8 c.f.u. per ml (usually 1 to 2 hours). A suitable aliquot of phage in saline phosphate buffer was added to the culture so that a concentration of 10^5 p.f.u. per ml and a multiplicity of 10^{-3} was achieved. This culture was incubated for 7 1/2 to 8 hours. Approximately 10 to 20 ml of chloroform were added, and the lysate stored at 4°C overnight. Chloroform was removed from the lysate by blowing air over the surface of it: 1 to 2 mg of pancreatic DNAse and $MgSO_4$ to 10^{-3} M were added and the lysate was incubated at 37°C for 1 hour to reduce the viscosity by digestion of extraneous DNA. Finally it was centrifuged in the G.S.A. rotor of the Servall RC2 centrifuge for 30 mins at 8,000 r.p.m. Under these conditions values of 1 to 2 x 10^{11} p.f.u. per ml were routinely obtained for 1 to 2 liters of crude lysate.

2. Concentration of phage from lysates.

A variety of methods was examined in order to find a procedure effective in precipitating the phage from the dilute lysate suspensions. The addition of an equal or larger volume of 95% ethanol in the cold, precipitated no more than

10% of the p.f.u.s of the lysate. Ammonium sufate added to the lysates in amounts giving 10 to 80% saturation according to the nomogram of Dixon (1953) did not result in the recovery of phage activity in precipitates which were formed. Effective precipitation of the phage was found, however, on adjustment of the pH of the lysate in the range 8.0 to 8.5 in the cold. The recovery of p.f.u.s varied from 5 to 40%. Essentially complete recovery of the phage was found in precipitates resulting from the addition of 470 gm of ammonium sulfate per liter (70% saturation) at pH 8.0 to 8.5.

The procedure adopted for the concentration of phage from the lysate suspensions was as follows. The lysate was chilled in an ice bath and the pH adjusted to 8.2 with NaOH. While stirring with a magnetic stirrer, 470 gm of ammonium sulfate were added per liter of lysate. When this was dissolved the pH was readjusted to 8.2 and the suspensions left to stand at 4°C overnight. The precipitate was sedimented in the G.S.A. rotor of the Servall centrifuge at 8,000 r.p.m. for 30 mins. The pellets obtained were resuspended in 1/10 of the original lysate volume in saline phosphate buffer.

3. <u>Purification of phage.</u>

In order to ascertain the value of purification procedures three criteria were employed. The first was the recovery of phage activity. The second criterion was the ratio of the UV absorbancy to the phage titer. The absorbancy at 260 mμ and at 280 mμ of a phage preparation titering 1 x 10^{12} p.f.u. per ml will be referred to as the

E260 mμ and the E280 mμ, respectively. The E260 mμ values for the DNA-containing phages G and T2 are 1.56 (Murphy and Philipson, 1962) and 3.6 (corrected by Murphy and Philipson, 1962, from the data of Hershey et al, 1953): these values were used as a guide in evaluating the course of purification. The spectral properties of different phage preparations to be described have not been corrected for light scattering since the removal of both extraneous light scattering and absorbing material was the object of purification. The E values for the most purified phage preparations are therefore not true absorbancy values. The third criterion of purity was the absence of particulate matter other than intact phage particles when preparations were examined by electron microscopy.

The suspension obtained after precipitation with ammonium sulfate contained large amounts of bacterial debris, some of which was removed by centrifugation at 8,000 r.p.m. for 30 mins. in the GSA rotor of the Servall centrifuge. The brown coloured supernatant obtained after centrifugation contained essentially all of the phage activity of the initial crude lysate and a typical preparation had the following properties:

$$E260\ m\mu = 11.5$$

$$E280\ m\mu = 7.0$$

$$\frac{260}{280}\ \text{ratio} = 1.6$$

The phage suspension was subjected to differential centrifugation. Experiments were performed to observe the effects

Figure 11

Procedure for differential centrifugation of crude phage suspensions

Ammonium sulfate precipitate, suspended in saline-phosphate buffer at 1/10 the original lysate volume

8,000 r.p.m. for 30 mins.

Pellet
(discarded)

Supernatant

15,000 r.p.m. for 15 mins.

Pellet
(discarded)

Supernatant

25,000 r.p.m. for 30 mins.

Pellet
suspended in 100 to 200
ml of saline-phosphate
buffer

Supernatant
(discarded)

10,000 r.p.m.
for 15 mins.

Pellet
(discarded)

Supernatant

25,000 r.p.m. for 30 mins.

Pellet
suspended in saline-phos-
phate buffer as before

Supernatant
(discarded)

10.000 r.p.m.
for 15 mins.

Supernatant

Pellet
(discarded)

25,000 r.p.m. for 30 mins.

<u>Final pellet</u>

Supernatant
(discarded)

Table I

The effect of low and high speed centrifugation on the absorbancy and phage titer of an ammonium sufate suspension

The "Millipore filtrate" of an ammonium sulfate suspension had a titer of 7.6 x 10^{11} p.f.u. per ml. Its absorption at 260 mμ was 13.2, at 280 mμ was 8.2, and the ratio of absorption at 260 mμ and 280 mμ was 1.6. 10 ml aliquots of this preparation were centrifuged as indicated and the pellets resuspended in 10 ml of saline-phosphate buffer.

Centrifugation procedure	Absorbancy 260 mμ	Absorbancy 280 mμ	Absorbancy $\frac{260\ m\mu}{280\ m\mu}$	Titer p.f.u./ml	% recovery of p.f.u. in high speed pellet
1. 10,000 r.p.m. for 15 mins.	0.4	0.25	1.6	5.4 x 10^{10}	
Snt: 25,000 r.p.m. for 30 mins.	2.9	2.1	1.38	7.4 x 10^{11}	94
2. 10,000 r.p.m. for 30 mins.	0.9	0.6	1.5	8.0 x 10^{10}	
Snt: 25,000 r.p.m. for 30 mins.	2.55	1.75	1.46	6.8 x 10^{11}	89
3. 15,000 r.p.m. for 15 mins.	1.4	1.0	1.4	1.9 x 10^{11}	
Snt: 25,000 r.p.m. for 30 mins.	2.1	1.4	1.5	4.7 x 10^{11}	71
4. 15,000 r.p.m. for 30 mins.	2.4	1.75	1.37	3.6 x 10^{11}	
Snt: 25,000 r.p.m. for 30 mins.	1.6	1.05	1.53	3.3 x 10^{11}	48

of speed and duration of sedimentation runs on the removal of impurities and the recovery of phage activity. The results of one experiment are shown in Table I. More than half of the 260 and 280 mμ absorbing material was removed from the suspension after sedimentation of the phage at 25,000 r.p.m. for 30 mins in the no. 40 rotor of the Spinco model L centrifuge. This absorbing material was predominantly RNA as it gave a positive orcinol reaction. A considerable amount of UV absorbing or light scattering material was removed by low speed centrifugation. It was evident too that as the speed and duration of low speed sedimentation runs was increased the recovery of p.f.u.s was correpondingly decreased. Bacterial debris in these preparations was visible in the electron microscope. Attempts to separate the debris from the phage by digestion with RNAse, DNAse, chymotrypsin, trypsin, or lysozyme in the presence of EDTA were unsuccessful. Chromatographic separation of the phage on DEAE- or CM-cellulose or on CM-Sephadex columns was unsuccessful because of inactivation which resulted from this treatment.

Differential centrifugation was incorporated in the purification procedure according to the scheme shown in Fig. 11. The final high speed pellet was suspended in saline-phosphate buffer. Further purification of this material was attempted by means of rate zonal centrifugation in a linear gradient of 5 to 40% sucrose. Using this technique bacterial debris was not completely dissociated from the phage

band which was very diffuse and from which about 50% of the activity was recovered. More favourable results were obtained by isopycnic gradient centrifugation in cesium chloride according to the method of Strauss and Sinsheimer (1963).

In the procedure adopted 12 ml of a phage suspension in saline-phosphate buffer, representing an initial 1 to 2 liters of lysate, were distributed between the three tubes of the S.W. 39 rotor of the Spinco model L centrifuge. Cesium chloride (Gallard Schlesinger: special biochemical grade) was added in the amount of 0.7 gm per ml of phage suspension and dissolved in the tubes. The density gradient was established during a run of 24 hours at 30,000 r.p.m. The rotor was allowed to decelerate without braking to ensure that the gradient was not disturbed. After centrifugation the phage appeared as a discrete band, about 1/16 of an inch deep, located about 1/3 of the distance up from the bottom of the centrifuge tube. Initial runs performed in the no. 40 fixed angle rotor according to Strauss and Sinsheimer did not yield such discrete bands. Visible impurities were found in the form of a tough brown plug at the top of the gradient, and in a pellet at the bottom of the tube. The phage was extracted by gently pushing the needle of a 5 ml syringe to the level of the phage band and withdrawing it. The phage bands from the three tubes were combined to give a volume of about 3 ml, which was then diluted to 50 or 100 ml with saline-phosphate buffer. The phage was freed of cesium chloride by sedimenting in the

Table III

Spectral properties of phage suspensions obtained by differential centrifugation (modified procedure) before and after centrifugation in cesium chloride

Prep. no.	Before centrifugation			After centrifugation			p.f.u. recovery from gradient %	p.f.u. total recovery %
	E260 mμ	E280 mμ	$\frac{E260\ m\mu}{E280\ m\mu}$	E260 mμ	E280 mμ	$\frac{E260\ m\mu}{E280\ m\mu}$		
6*	-	-	-	3.75	2.31	1.61	50	45
7*	4.25	2.92	1.46	2.4	1.5	1.60	78	67

* S.W. 39 rotor used for cesium chloride centrifugations

Figure 12

The modified differential centrifugation procedure used for purification of phage from ammonium sulfate suspensions

Ammonium sulfate suspension

8,000 r.p.m. for 30 mins.

Pellet (discarded)

Supernatant

10,000 r.p.m. for 10 mins.

Pellet (discarded)

Supernatant

25,000 r.p.m. for 30 mins.

Supernatant (discarded)

Pellet suspended in saline phosphate buffer

Cesium chloride density gradient

Table II

Spectral properties of phage suspensions before and after centrifugation in cesium chloride

Prep. no.	Before centrifugation			After centrifugation			p.f.u. recovery from gradient %	p.f.u. total recovery %
	E260 mμ	E280 mμ	$\frac{E260\ m\mu}{E280\ m\mu}$	E260 mμ	E280 mμ	$\frac{E260\ m\mu}{E280\ m\mu}$		
1*	4.8	3.6	1.38	2.10	1.35	1.56	87	45
2*	3.2	2.3	1.39	2.79	1.82	1.52	53	25
3*	4.5	3.2	1.40	2.57	1.62	1.57	84	34
4**	6.8	5.4	1.28	4.36	2.90	1.51	53	21
5**	5.9	4.8	1.20	2.68	1.79	1.51	94	35

* No. 40 rotor used for cesium chloride centrifugation.

** S.W. 39 rotor used for cesium chloride centrifugation.

no. 40 rotor of the model L Spinco at 30,000 r.p.m. for 30 mins. and resuspending in the desired buffer.

The properties of some preparations before and after purification in cesium chloride are shown in Table II. All absorbancy values both before and after centrifugation in cesium chloride are adjusted to correspond with a titer of 1×10^{12} p.f.u. per ml. Little if any debris is visible on examination of these preparations in the electron microscope.

The purification procedure, comprising the scheme shown in Fig. 11, followed by centrifugation in cesium chloride, gave recoveries of 20 to 50% of the p.f.u. contained in the ammonium sulfate suspensions. Considerable losses of phage occurred in the intermediate low speed centrifugation runs. Experiments were therefore performed to check the degree of purity obtained when some of these intermediate runs were omitted. Two lysates were produced and the ammonium sulfate suspensions were treated as shown in Fig. 12. The properties of the phage suspensions obtained after this treatment are shown in Table III.

The two preparations obtained by the modified purification procedure appeared to have spectral properties comparable to those obtained by the longer procedure and the recovery of phage activity appeared to be higher. Chemical studies on preparations obtained by both procedures are described in Chapter 3, Part B.

Table V

DNA contents of phage SDI preparations estimated by the diphenylamine method

Prep no	Experimental values = µg DNA/ml phage prep	Average value µg DNA/ml of prep	Titer of phage prep p.f.u./ml	DNA content adjusted to titer of 10^{12} p.f.u./ml = µg DNA/ 10^{12} p.f.u.	µg DNA/10^{12} $\frac{\text{p.f.u.}}{\text{E260}}$
1	88 96	92	1×10^{12}	92	44
4	126 129	128	8.6×10^{11}	149	34
5	117 125	121	1.4×10^{12}	86	32
6	134 132	133	1.1×10^{12}	121	33
7	151 157	154	1.5×10^{12}	103	43
Average value: µg DNA per 1×10^{12} p.f.u.:				110	

Table IV

The size of phage SDI determined from electron micrographs

Dimensions	Head	Tail
Diameter ($m\mu$)	50	6.2
Length ($m\mu$)	-	188
Volume ($m\mu^3$)	65.5×10^3	5.7×10^3
Total volume: Head + tail	71.2×10^3	

Figure 13

Electron micrograph of phage SDI (x 260,000)

Chapter 3.

B. Results of Chemical and Physical Studies on Phage SDI.

1. Morphology.

Phage SDI possesses a well defined head and tail, visible in the electron micrograph of Fig. 13. The head has a regular hexagonal outline characteristic of phages possessing icosahedral symmetry. The tail appears to be of a simple type having no sheath or apparent contractile mechanism. Tail fibres or other terminal appendages have not been observed in electron microscope examinations of various phage preparations. The tail possesses a uniform helical pattern suggesting that it is composed of regularly arranged protein sub-units. The dimensions of the phage were determined from electron micrographs and are shown in Table IV.

2. Nucleic acid composition.

Phage SDI contains DNA. The results of diphenylamine determinations made on five different phage preparations are shown in Table V. The phage preparations are described in Tables II and III. The DNA content was obtained by equating the moles of total deoxyribose found with the mole average nucleotide weight of the constituent deoxyribonucleotides. This was calculated from the relative proportion of these in SDI DNA (Part B, 6). The average DNA content is 110 μg of DNA per 10^{12} p.f.u., or 1.1. x 10^{-16} gm per p.f.u. There is a considerable variation in the estimated DNA content of the phage in different preparations. This may be attributed tothe presence of inactive phage

Table VI

Phosphorus content of whole phage SDI preparations

No of prep	Experimental value µg P/ml phage prep		Average value µg P/ml prep	Titer of phage prep p.f.u./ml	P content adjusted to titer of 1×10^{12} p.f.u.=µg P/1×10^{12} p.f.u.	Phosphorus as % by wt of DNA
1	6.6	6.5	6.55	1×10^{12}	6.55	7.15
4	9.58	9.4	9.49	8.6×10^{11}	11.0	7.1
5	8.8	9.0	8.9	1.4×10^{12}	6.35	7.25
6	9.8	9.8	9.8	1.1×10^{12}	8.9	7.4
7	11.1	11.0	11.05	1.5×10^{12}	7.35	7.23
Average of values:					8.03	7.23

particles in varying amounts in the different preparations. Such inactive particles would not contribute to an estimation of the plaque forming units present in a preparation. They would however contribute to the DNA, phosphorus, and nitrogen estimations of the preparation. This is illustrated by the fact that preparations having a high E 260 mμ and low recovery after centrifugation in cesium chloride (preparations 4 and 6 of Tables II and III) also have high DNA contents relative to their titer. Other contaminations may be present however, since the ratio $\frac{\mu g \text{ DNA per } 10^{12} \text{ p.f.u.}}{\text{E } 260 \text{ m}\mu}$ is not constant in all the preparations as would be expected if intact but biologically inactive particles were contributing to both determinations.

3. Phosphorus content.

Total phosphorus was estimated in five phage preparations described in Tables II, III and V. The values obtained are recorded in Table VI. The suspending medium of the phage preparations was analysed after precipitation of the phage and was found to be essentially free of phosphorus. The average phosphorus content of the phage preparations was found to be 8.03μg per 10^{12} p.f.u. from which a value of 8 x 10^{-18} gm per p.f.u. may be calculated. The same type of variation previously described for the diphenylamine estimations of different phage preparations was found in the phosphorus estimations. In contrast to the discrepancy in the absolute content of DNA and phosphorus among different phage preparations, the ratio of the phosphorus content to the DNA content is nearly constant in all the preparations. This

FIG. 14

The specific gravity and phage titer (p.f.u./ml) of fractions collected from a cesium chloride density gradient.

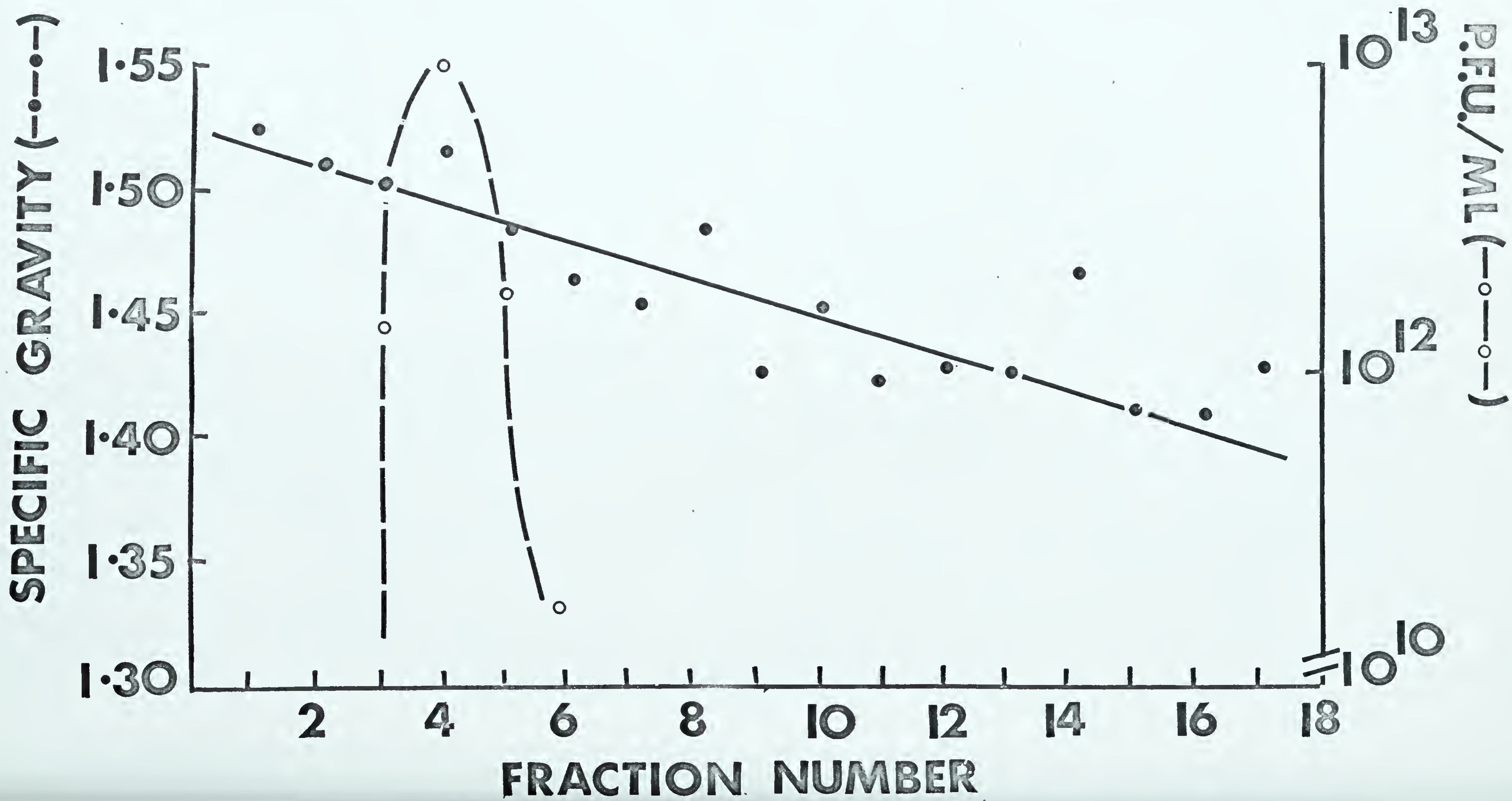


Table VIII

The buoyant density of phage SDI

Expt. no.	Buoyant density gm/cm^3
1	1.55
2	1.49
3	1.51
mean	1.52 ± 0.03

Table VII

The nitrogen content of phage SDI

The estimations were made on preparation 7 of Table V which titered 1.5 x 10^{12} p.f.u. per ml

Estimation no	Fraction of phage taken	Length of digestion (hrs)	N found (μg/ml)	Total	Average value for whole phage (μg/ml)	Average value for nucleic acid fraction (μg/ml)	Average value for protein fraction (μg/ml)
1	whole phage	16	45	-			
2	"	24	39*	-			
3	"	6	39*	-			
4a	whole phage	16	40				
b	nucleic acid	16	22.7	41.9			
c	protein	16	19.2				
5a	nucleic acid	16	24.2	42.4			
b	protein		18.2				
					42.5	23.5	18.7

* Not used in calculation of average value

lends support to the interpretation of the variability in both determinations being due to the presence of variable amounts of inactive particles in the different preparations. Phosphorus accounts for 7.23% of the weight of DNA.

4. Nitrogen content.

The nitrogen content of whole phage and of the phage protein and nucleic acid fraction of preparation 7 (described in Tables III, V and VI) was determined. An average value of 42.5 μg of nitrogen per 1.5×10^{12} p.f.u. or 2.83×10^{-17} gm per p.f.u. was obtained (Table VII). The protein nitrogen contributes only 45% of the total phage nitrogen, while the nucleic acid fraction, that fraction soluble in hot trichloroacetic acid, contributes about 55% of the total nitrogen.

5. Buoyant density.

The density of phage SDI was determined from the specific gravity of the phage fraction in a cesium chloride density gradient. A typical plot of specific gravity and phage titer versus fraction number (each fraction contained 10 drops) is shown in Figure 14. The results of three density determinations are shown in Table VIII. The mean density was found to be 1.52 gm per cm^3.

6. Bases analyses of DNA.

The base composition of SDI DNA was determined by analysing the products of perchloric acid hydrolysis of whole phage and of enzymic hydrolysis of the DNA. SDI DNA was found to contain adenine, thymine, guanine, and cytosine.

Table XIII

Mobilities of bases obtained by perchloric acid hydrolysis of phage SDI during chromatographic separation under conditions described in Chapter 2, Part B6. The mobilities are calculated relative to thymine

Expt	$\frac{T}{T}$=1	$\frac{T}{C}$	$\frac{T}{A}$	$\frac{T}{G}$	$\frac{T}{T}$=1	$\frac{T}{C}$	$\frac{T}{A}$	$\frac{T}{G}$
	Values of standards				Values of hydrolysates			
1	-	1.61	2.34	3.21	-	1.64	2.30	3.30
2	-	1.58	2.17	3.10	-	1.58	2.20	3.10
3	-	1.59	2.3	3.20	-	1.62	2.30	3.50
4	-	1.49	2.18	2.89	-	1.49	2.18	2.92
5	-	1.56	2.33	3.14	-	1.61	2.34	3.25
6	-	1.65	2.30	3.26	-	1.68	2.30	3.20

Table XII

Spectral properties of the chromatographic eluates corresponding to guanine in 0.1 N HCl

Expt	λ 250mμ	λ 260mμ	250mμ/260mμ	λ 280mμ	λ 260mμ	280mμ/260mμ	λ 290mμ	λ 260mμ	290mμ/260mμ
guanine	0.760	0.550	1.382	0.440	0.550	0.800	0.250	0.550	0.454
1	0.440	0.330	1.330	0.260	0.330	0.788	0.100	0.330	0.303
2	0.380	0.285	1.333	0.220	0.285	0.772	0.100	0.285	0.351
3	0.375	0.275	1.364	0.210	0.270	0.778	0.100	0.270	0.370
4	0.400	0.300	1.338	0.225	0.300	0.750	0.122	0.300	0.406
5	0.460	0.340	1.350	0.250	0.340	0.730	0.129	0.340	0.378
6	0.220	0.160	1.375	0.124	0.160	0.776	0.078	0.160	0.480
7	0.300	0.220	1.364	0.175	0.220	0.795	0.103	0.220	0.468
8	0.350	0.260	1.346	0.210	0.260	0.808	0.115	0.260	0.444
9	0.295	0.220	1.341	0.174	0.220	0.792	0.110	0.220	0.500
10	0.385	0.280	1.375	0.217	0.280	0.775	0.135	0.280	0.484
Average			1.352			0.776			0.418

Table XI

Spectral properties of the chromatographic eluates corresponding to adenine in 0.1 N HCl

Expt	λ 250mμ	λ 260mμ	$\frac{250m\mu}{260m\mu}$	λ 280mμ	λ 260mμ	$\frac{280m\mu}{260m\mu}$	λ 290mμ	λ 260mμ	$\frac{290m\mu}{260m\mu}$
adenine	0.395	0.512	0.771	0.175	0.512	0.342	0.013	0.512	0.025
1	0.345	0.450	0.771	0.178	0.450	0.395	0.020	0.450	0.044
2	0.360	0.460	0.783	0.119	0.460	0.259	0.050	0.460	0.109
3	0.300	0.390	0.769	0.105	0.390	0.269	0.010	0.390	0.026
4	0.385	0.485	0.794	0.120	0.485	0.247	0.015	0.485	0.024
5	0.365	0.465	0.787	0.14	0.465	0.301	0.025	0.465	0.054
6	0.175	0.225	0.778	0.070	0.225	0.311	0.010	0.225	0.044
7	0.270	0.340	0.794	0.119	0.340	0.350	0.015	0.340	0.044
8	0.275	0.360	0.764	0.110	0.360	0.306	0.005	0.360	0.140
9	0.245	0.315	0.778	0.115	0.315	0.365	0.020	0.315	0.063
10	0.315	0.400	0.788	0.120	0.400	0.300	0.012	0.400	0.030
Average			0.780			0.310			0.045

Table X

Spectral properties of the chromatographic eluates corresponding to deoxythymidine-5'-phosphate in 0.1 N HCl

Expt	λ 250mμ	λ 260mμ	$\frac{250m\mu}{260m\mu}$	λ 280mμ	λ 260mμ	$\frac{280m\mu}{260m\mu}$	λ 290mμ	λ 260mμ	$\frac{290m\mu}{260m\mu}$
5'-TMP	0.395	0.630	0.627	0.450	0.630	0.714	0.130	0.630	0.206
1	0.220	0.325	0.677	0.245	0.325	0.754	0.090	0.325	0.277
2	0.165	0.259	0.637	0.189	0.259	0.765	0.051	0.259	0.197
3	0.162	0.255	0.635	0.195	0.255	0.765	0.06	0.255	0.232
4	0.165	0.275	0.600	0.225	0.275	0.818	0.075	0.275	0.273
5	0.180	0.275	0.635	0.235	0.273	0.855	0.095	0.275	0.345
6	0.085	0.142	0.599	0.112	0.142	0.789	0.030	0.142	0.207
7	0.149	0.218	0.683	0.109	0.218	0.500	0.038	0.218	0.174
8	0.120	0.210	0.571	0.155	0.210	0.738	0.035	0.210	0.167
9	0.120	0.190	0.632	0.135	0.190	0.711	0.040	0.190	0.211
10	0.155	0.250	0.620	0.185	0.250	0.740	0.055	0.250	0.220
Average			0.631			0.744			0.230

Table IX

Spectral properties of the chromatographic eluates corresponding to deoxycytidine-5'-phosphate in 0.1 N HCl

Expt	λ			λ			λ		
	250mμ	260mμ	250mμ/260mμ	280mμ	260mμ	280mμ/260mμ	290mμ	260mμ	290mμ/260mμ
5'-dCMP	0.180	0.425	0.423	0.895	0.425	2.106	0.655	0.425	1.541
1	0.110	0.238	0.462	0.530	0.238	2.226	0.395	0.238	1.660
2	0.082	0.210	0.390	0.465	0.210	2.214	0.345	0.210	1.640
3	0.099	0.220	0.450	0.460	0.220	2.091	0.340	0.220	1.619
4	0.090	0.229	0.393	0.520	0.229	2.271	0.390	0.229	1.703
5	0.090	0.215	0.419	0.490	0.215	1.902	0.390	0.215	1.814
6	0.040	0.105	0.381	0.195	0.105	1.857	0.130	0.105	1.238
7	0.085	0.195	0.435	0.400	0.195	2.051	0.285	0.195	1.462
8	0.095	0.210	0.452	0.420	0.210	2.000	0.300	0.210	1.429
9	0.090	0.215	0.419	0.465	0.215	2.163	0.350	0.215	1.628
10	0.060	0.145	0.414	0.335	0.145	2.310	0.250	0.145	1.724
Average			0.422			2.109			1.592

The bases were identified spectrophotometrically by comparison with known standards. Spectral properties of the products of enzymic hydrolysis of DNA and of standard compounds are presented in Tables IX to XII. Similar data were obtained for the bases released on perchloric acid hydrolysis, using standard bases for comparision. The mobility of the products of hydrolysis relative to the mobility of known compounds in the chromatographic systems used was also employed as a means of identification. The mobilities of the bases released by perchloric acid hydrolysis of the phages are documented in Table XIII: the mobilities are expressed relative to that of thymine.

The appearance of adenine and guanine in the products of enzymic hydrolysis is due to hydrolysis of the N-C glycosidic bond of the purine nucleotides in the 0.1 N HCl used to elute the nucleotides from the chromatograph. Hydrolysis was 98 to 99% complete in 1 hour for both 5'-dGMP and 5'-dAMP.

In the first five enzymic experiments a period of one hour was used for the elution and hydrolysis. In the last five experiments the 5'-dGMP and 5'-dAMP were left in 0.1 N HCl overnight at 37°C to ensure 100% hydrolysis. No differences were found in the results obtained under the two conditions of elution.

The molar base ratios were estimated from the relative amounts of each of the products released by hydrolysis. Quantitation was based on the molar extinction coefficients of compounds at the wavelengths of maximum absorption, λ max, in 0.1 N HCl. In the case of enzymic hydrolysis the products in 0.1 N HCl were the purine bases and the pyrimidine deoxyribonucleotides.

Table XV

Molar base ratios of phage SDI DNA obtained by analysis of perchloric acid hydrolysates of whole phage

Estimation no	Molar base ratios calculated to total 4.00				% recovery
	Adenine	Cytosine	Thymine	Guanine	
1	0.95	1.15	0.88	1.02	84
2	0.98	1.14	0.82	1.06	79
3	0.96	1.13	0.86	1.05	80
4	1.01	1.08	0.84	1.07	76
5	0.97	1.10	0.89	1.04	78
6	1.03	1.10	0.87	1.00	88
Average values	0.98	1.11	0.87	1.04	81

Table XIV

Base ratios (brought to molar total of 4) obtained by analysis of enzymic hydrolysates of phage SDI DNA

Expt no	Adenine	Cytosine	Guanine	Thymine	% recovery for expt	% 260mμ OD left at origin
1	0.948	1.060	1.006	0.986	-	2.6
2	1.035	1.057	0.988	0.920	-	1.4
3	0.929	1.095	1.023	0.952	-	0
4	1.013	1.087	0.973	0.926	-	0.8
5	0.972	1.015	1.100	0.913	-	0.8
6	0.947	1.045	1.053	0.955	89	0
7	0.936	1.139	0.989	0.936	96	1.3
8	0.948	1.107	1.072	0.872	100	1.5
9	0.969	1.069	1.052	0.910	92	0
10	0.945	1.109	1.040	0.906	94	1.0
total	9.642	10.783	10.296	9.276		
average	0.964	1.078	1.030	0.928	94	0.8

The extinction coefficients used were:

A	$\varepsilon = 13.2 \times 10^3$	at 263 mμ
G	" $= 11.4 \times 10^3$	" 248 mμ
5'-dCMP	" $= 13.2 \times 10^3$	" 286 mμ
5'-dTMP	" $= 9.6 \times 10^3$	" 267 mμ

The amount of each compound isolated was calculated according to the equation:

$$\frac{\text{absorbancy at } \lambda \text{ max.}}{\text{molar extinction at } \lambda \text{ max.}} = \text{moles per liter.}$$

The relative amount of each compound in each analysis was estimated by using the equation:

$$\frac{\text{moles of compound} \times 4}{\text{sum (in moles) of 4 constituent compounds}} = \text{molar ratio in a total of 4 moles}$$

Similar calculations were made to determine the molar base ratios in perchloric acid hydrolysates: in this case the molar extinction coefficients of the bases were used in the calculation. The molar base ratios determined for each of 10 analyses of enzymic hydrolysates, and for 6 analyses of acid hydrolysates are shown in Tables XIV and XV. The molar base ratios obtained indicate a near equivalence in the amounts of all 4 bases in the DNA. The recovery of nucleotide components in both types of analysis was calculated from the phosphorus content of the preparation analysed assuming a molar relationship between the two. Enzymic digestion appeared to be complete since virtually no UV absorbing material was detected at the origin of any of the chromatograms as would be expected if oligonucleotides

Table XVI

Statistical analysis for the true mean proportion of adenine in SDI DNA

Molar ratio (x_1)	Molar ratio^2 $(x_1)^2$
0.948	0.8987
1.035	1.0710
0.929	0.8630
1.013	1.0260
0.972	0.9448
0.947	0.8968
0.936	0.8761
0.948	0.8987
0.969	0.9390
0.945	0.8930
$9.642 = \Sigma x^1$	$9.3071 = (\Sigma x_1^2)$
$92.9681 = (\Sigma x_1)^2$	
$0.9642 = \bar{x}$	

$$Sx^2 = \frac{1}{n-1}\left[\Sigma x_1^2 - \frac{1}{n}(\Sigma x_1^2)\right]$$

$$= 0.00114$$

$$\therefore Sx = 0.03376$$

The true mean proportion, μ, with 95% confidence limits for 10 observations is calculated from:

$$\mu = \bar{x} \pm t_{95,n-1}\left[\frac{S_x}{\sqrt{n-1}}\right] \qquad t_{95,9} = 2.262$$

$$\mu = 0.9642 \pm 0.02552$$

were present in the digests. The values for thymine obtained from the analyses of acid hydrolysates were consistently low. A loss of thymine during hydrolysis with perchloric acid has been noted by other workers, e.g. Marshak and Vogel (1951).

Only the data obtained for the enzymic hydrolysates were analysed statistically because the results were considered more reliable than those obtained from the acid hydrolysates. The mean proportion of the 4 bases in SDI DNA is:

adenine	0.964 ± 0.026
cytosine	1.078 ± 0.027
guanine	1.030 ± 0.031
thymine	0.928 ± 0.024

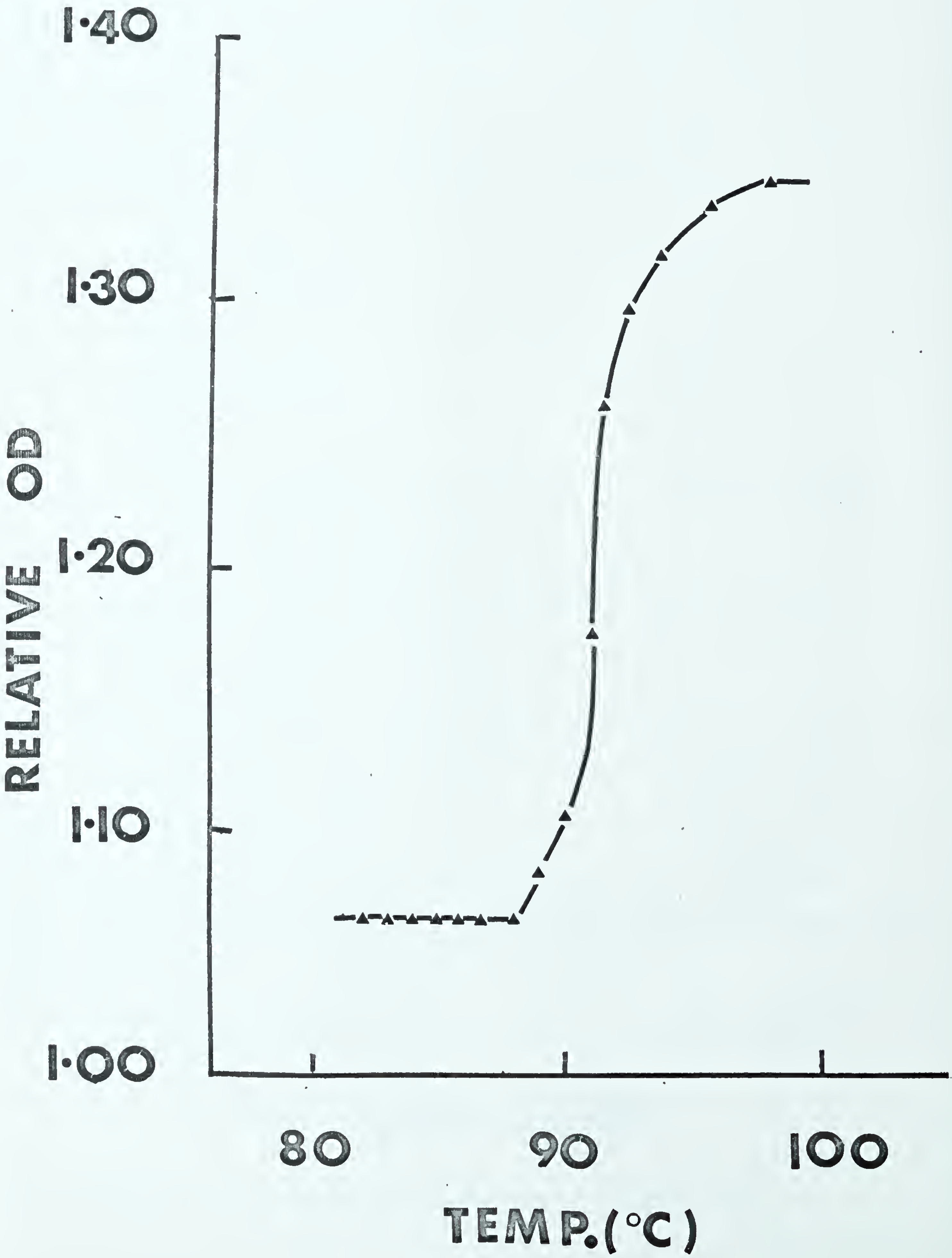
The statistical analysis for adenine is given as an example of the statistical method used to calculate the true mean proportion (μ) with 95% confidence (Table XVI). Although the 4 bases occur in almost equimolar proportions the true mean proportion of adenine is similar to that of thymine and different from that of guanine and cytosine. The true mean proportion of guanine is similar to that of cytosine. The molar equivalence of adenine and thymine and of guanine and cytosine is analogous to that found in most DNAs (Chargaff, 1955). The sum of the purine bases is essentially equivalent to the sum of the pyrimidine bases:

$$\frac{\text{adenine + guanine}}{\text{thymine + cytosine}} = 0.994$$

In order to determine whether the phage DNA contained

FIG 15.

The thermal denaturation of phage SDI DNA, determined by the changes in absorption at 260 mu.



5-methylcytosine or 5-hydroxymethylcytosine as minor constituents, formic acid hydrolysates were chromatographed in two solvent systems known to resolve these bases from the 4 bases found in SDI DNA. Neither base was found: in the case of 5-methylcytosine, 1 mole in 50 moles of the DNA bases would have been detected. A similar consideration applies to 5-hydroxymethylcytosine although this was not run as a control. Uracil or uridylic acid was not detected on any of the chromatograms.

SDI DNA was hydrolysed in HCl in order to release any glucose that might be associated with the bases. No glucose was found although 1 mole in 30 moles of DNA bases would have been detected.

The thermal denaturation of SDI DNA was examined by following the change in absorption at 260 mμ. A "melting curve" is shown in Fig. 14: the absorbancy relative to that at 25°C is plotted over the range 25 to 100°C. The sharp hyperchromic change over a narrow temperature range, in this case 89 to 96°C, and the amount of hyperchromicity, 28%, are characteristic of highly ordered double stranded structures (Shack, 1958; Sinsheimer, 1959).

The temperature at which half the maximal absorption of the DNA was reached (Tm) is linearly related to the guanine + cytosine content of the DNA (Marmur and Doty, 1962). In two experiments the Tm of SDI DNA was found to be 91.3°C and 91.1°C from which a figure for the guanine + cytosine content of 53.2% can be estimated. This is in good agreement with the figure of 52.7% obtained by base analysis of the DNA.

Chapter 4.

Discussion and Summary.

Chapter 4.

Discussion.

Phage SDI is similar in size (Table IV) and morphology to the coliphages λ and T1. Phage λ has a head 54 $m\mu$ in diameter, and a sheathless tail 7 x 140 $m\mu$ in length; T1 has a head 50 $m\mu$ in diameter, and a similar tail 10 x 150 $m\mu$ long. The regular hexagonal outline of the SDI head suggests icosahedral symmetry which is possessed by other sheathless tailed phages such as T5 (Bradley, 1963). The chemical composition of phage SDI will be mainly compared to that of the <u>Bacillus</u> <u>megatherium</u> phage G (Murphy and Philipson,1962) which is slightly larger than SDI but also of a similar morphological appearance. The chemical and physico-chemical properties of phage G studied by Murphy and Philipson provide more uniform parameters for comparison with phage SDI than the data available on phages λ and T1.

The purification procedures outlined in Chapter 3, Part A, have yielded a preparation of phage SDI (preparation 7, Table III) having a $\frac{P}{N}$ ratio of 0.26 which can be compared to that of 0.32 for phage G, and 0.27 for T5 (Smith and Wyatt, 1951). The $\frac{P}{N}$ ratio is characteristic of the nucleoprotein composition of these phages.

The DNA component of phage SDI contains essentially equimolar amounts of adenine and thymine, and of guanine and cytosine. It is thus similar to cellular DNA and to many other bacteriophages. It seems probable that SDI DNA

possesses an extensively ordered structure since thermally induced denaturation takes place over a narrow temperature range and results in a 28% increase in absorbancy at 260 mμ. This absorbancy increase is similar to the value of 25% obtained by Lawley (1956) and Shack (1958) in similar studies of calf thymus DNA. It is in contrast to the results obtained for single stranded Ø x 174 DNA or for samples of previously denatured DNA (Sinshiemer, 1959). The thermal denaturation profile together with the equivalence of the base pairs suggests that SDI DNA possesses a structure of the type proposed for DNA by Watson and Crick (1953).

The purification procedure has not yielded uniform phage preparations as evidenced by the figures obtained for the E260 mμ values and the DNA and phosphorus contents relative to the titer of each preparation. Several preparations were examined by electron microscopy and found to be essentially free of particulate debris. Since the phage has been shown to have a buoyant density of 1.52 gm/cm^3, and protein and native double stranded DNAs have buoyant densities of approximately 1.3 gm/cm^3 and 1.7 gm/cm^3 respectively (Weigle and Meselson, 1959) it seems unlikely that either protein or DNA would band with the phage during centrifugation in cesium chloride. Ghosts, phage protein coats without the nucleic acid portion, would not be expected to band with the intact phage during centrifugation but they could arise after centrifugation. Thus Murphy and Philipson note that their best preparations of phage G contained only 3% of ghosts after chromatography on DEAE-cellulose. After 2 weeks storage at $4^{\circ}C$ however the fraction of ghosts had risen to 20%. In

the preparations of SDI phage which have been examined in the electron microscope, the approximate proportions of ghosts have varied between 1 or 2% to about 20% in different preparations. Except for preparation 2, Table II, preparations having a high E260 mμ value after purification are those which lost considerable titer during centrifugation in cesium chloride. These preparations also had a high DNA content relative to their titer. It would appear that these preparations contained higher numbers of biologically inactive particles but retained some or all of their nucleic acid component. It is noteworthy that the recovery of phage G after centrifugation in cesium chloride is complete in terms of absorbancy units but only 65% with respect to phage activity (Murphy and Philipson, 1962). The lowest E260 mμ value for phage SDI preparations was 2.1 (prep. no. 1, Table II). The volume of SDI was estimated to be 72.1×10^3 $m\mu^3$. By comparison phage G whose volume has been estimated to be 98.3×10^3 $m\mu^3$ has an E260 mμ value of 1.53 (Murphy and Philipson, 1962). Apparently all the SDI preparations contain inactive particles.

The nitrogen determinations made on preparation 7, Table III, indicate that 55% of the total phage nitrogen is contributed by the DNA fraction and 45% by the protein fraction. This result is analogous to that found for the coli phage T2 in which 53% of the total nitrogen is contributed by the DNA fraction and 40% by the protein fraction (Herriott and Barlow, 1952). In the case of T2 some 6 to 7% of the nitrogen is also contributed by material soluble in cold trichloroacetic acid.

A knowledge of the nitrogen contribution of the nucleic acid and protein fractions of preparation 7 permits an estimate to be made of the relative amounts of protein and DNA in the phage. The nitrogen content of the protein fraction can be calculated by assuming that nitrogen comprises 16% of the protein weight:

$$18.7 \times \frac{100}{16} = 116.9 \ \mu g \text{ protein per ml.}$$

The nitrogen content of the nucleic acid fraction was 23.5 μg per ml: the weight of the DNA fraction can be estimated by utilizing the nitrogen contribution to the mole average nucleotide weight of the DNA calculated from its base composition:

$$23.5 \times \frac{324.6}{52.9} = 144.2 \ \mu g \text{ DNA per ml.}$$

This figure is somewhat less than the 154 μg per ml obtained by the diphenylamine assay. Based on these estimates, the DNA component comprises 55%, and the protein component 45% of the total weight of the phage. Phage G contains 55% by weight of DNA when a figure for the partial specific volume is utilized and 45% by weight of DNA based on its phosphorus content (Murphy and Philipson, 1962).

The phosphorus content of the phage accounts for 7.2% of the DNA weight. A value of 9.6% would be expected on the basis of the nucleotide composition and the diphenylamine estimation of the DNA content. The reason for this discrepancy is not immediately apparent. The phosphorus content of preparation 7 was 11.05 μg per ml accounting for 7.7% of the DNA weight and 4.3% of the whole phage

weight estimated from the nitrogen analyses. Phosphorus accounts for 4.9% of the weight of phage G (Murphy and Philipson, 1962), and 5.2% of the weight of phage T2 (Herriott and Barlow, 1952). Murphy and Philipson acknowledged that their figure was low and suggested that phage particles might have been lost by adsorption to the dialysis membranes used. In the case of SDI, phage activity was not lost on dialysis against 1% ammonium acetate.

An approximate calculation of the mass of phage SDI can be made on the basis of its dimensions and its buoyant density:

$$71.2 \times 10^{-21}\ \mathrm{cm^3/particle} \times 1.52\ \mathrm{gm/cm^3} = 1.08 \times 10^{-16}\ \mathrm{gm/particle.}$$

The mass of the phage can also be estimated from the values obtained for the nitrogen assay of preparation 7; the average total nitrogen of the preparation was found to be 4.25 μg/1.5 x 10^{12} p.f.u. The contributions of the DNA and protein fractions to the total weight were calculated as previously outlined from the percentage contribution of nitrogen to these fractions. The calculation for the mass is as follows:

$$\begin{aligned} 1.5 \times 10^{12}\ \mathrm{p.f.u.} &= 117\ \mu\mathrm{g\ protein} + 144\ \mu\mathrm{g\ DNA} \\ &= 261 \times 10^{-6}\ \mathrm{gm} \\ 1\ \mathrm{p.f.u.} &= 1.74 \times 10^{-16}\ \mathrm{gm} \end{aligned}$$

The large discrepancy between the two estimations of the mass of phage SDI can be attributed in part to the dependence on phage activity as a parameter in the calculation based on the nitrogen assay.

Chapter 4.

Summary.

Phage SDI possesses a head of regular hexagonal outline and a long sheathless tail. The head is 50 mμ in diameter and the tail is 6.2 mμ wide, and 188 mμ long.

Phage SDI has been purified from lysates titering 1 to 2 x 10^{11} p.f.u. per ml. The purification procedure involved concentration of the phage from the lysates with ammonium sulfate at pH 8.2, differential centrifugation and density gradient centrifugation in a cesium chloride solution. The yield of phage varied between 20 and 70%. The preparations obtained were judged to be free of contamination when inspected in the electron microscope.

Five preparations were analysed with respect to their UV absorption and DNA and phosphorus contents relative to their titer. Discrepancies in these analyses could be attributed to the presence of varying amounts of biologically inactive particles in the different preparations.

The average DNA content was found to be 1.1 x 10^{-16} gm per p.f.u. and the phosphorus content 8 x 10^{-18} gm per p.f.u. The nitrogen content was determined for one phage preparation and found to be 2.8 x 10^{-17} gm per p.f.u., of which 55% was contributed by the DNA fraction and 45% by the protein fraction. DNA comprises approximately 55% and protein 45% of the total weight of the phage.

Base analysis of SDI DNA revealed that it contains almost equimolar amounts of adenine and thymine and of

guanine and cytosine. Guanine and cytosine comprise approximately 53% of the DNA bases. No 5-methylcytosine, 5-hydroxymethylcytosine or glucose was detected. SDI DNA is a highly ordered structure since thermal denaturation results in a sharp increase in UV absorption over a narrow temperature range.

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B29831